Radial Equilibrium Lengths of Actomyosin Cross-Bridges in Muscle

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ABSTRACT Radial equilibrium lengths of the weakly attached, force-generating, and rigor cross-bridges are determined by recording their resistance to osmotic compression. Radial equilibrium length is the surface-to-surface distance between myosin and actin filaments at which attached cross-bridges are, on average, radially undistorted. We previously proposed that differences in the radial equilibrium length represent differences in the structure of the actomyosin cross-bridge. Until now the radial equilibrium length had only been determined for various strongly attached cross-bridge states and was found to be distinct for each state examined. In the present work, we demonstrate that weakly attached cross-bridges, in spite of their low affinity for actin, also exert elastic forces opposing osmotic compression, and they are characterized by a distinct radial equilibrium length (12.0 nm vs. 10.5 nm for force-generating and 13.0 nm for rigor cross-bridge). This suggests significant differences in the molecular structure of the attached cross-bridges under these conditions, e.g., differences in the shape of the myosin head or in the docking of the myosin to actin. Thus, the present finding supports our earlier conclusion that there is a structural change in the attached cross-bridge associated with the transition from a weakly bound configuration to the force-generating configuration. The implications for imposing spatial constraints on modeling actomyosin interaction in the filament lattice are discussed.

INTRODUCTION

Several theories of the mechanism of muscle contraction hypothesize that the process of force generation is associated with structural changes in the actomyosin cross-bridges attached to actin. One of the models suggests that a structural change in the attached cross-bridge involves a transition from an initially weakly bound configuration to a strongly bound configuration (White and Taylor, 1976; Eisenberg and Greene, 1980; Eisenberg and Hill, 1985). Structural evidence for this model was first reported from equatorial x-ray diffraction studies (Brenner and Yu, 1993). The equatorial diffraction patterns from relaxed muscle fibers with most of the cross-bridges weakly attached to actin were different from those of fibers with strongly bound cross-bridges while generating isometric force. This suggested a structural difference between the two modes of attachment. This interpretation required the assumption that the observed changes are not mainly the result of changes in the fraction of cross-bridges attached to actin under both conditions. Although this assumption is supported by biochemical data obtained under similar conditions (Chalovich and Eisenberg, 1982), the conclusion would be strengthened if an alternative technique could be found that allows the detection of structural differences independent of the number of attached cross-bridges.

It might be argued that models for the weak to strong transition can be developed based on the atomic structures of myosin subfragment 1 (S1) (Rayment et al., 1993b) and actin monomers. Models, such as that proposed by Rayment et al. (Rayment et al., 1993a; Schroder et al., 1993), have provided working hypotheses for the mechanism of muscle contraction at the atomic scale. These models were constructed with the atomic structures of actin and S1 fitted into the density envelope derived by cryoelectron microscopy (Milligan et al., 1990) of actin filaments saturated with S1. The latter provided the constraints for orienting the atomic model of S1 on the model of the actin filament. However, S1 bound to actin filaments is not in a state of force generation, because the isolated S1 cannot be strained or deformed for sustaining tension. Moreover, the structure of S1 bound to actin in rigor may not be identical to the contracting state occurring in muscle fibers (Brenner and Yu, 1993).

We have recently developed a method that 1) can distinguish structural differences of the actomyosin cross-bridges independently of the fraction of attached cross-bridges, and 2) provides, for radially unstrained cross-bridges, an estimate of the distance between the surface from which the cross-bridge projects from the thick filament backbone and the surface of the thin filament, where the attachment occurs (Brenner and Yu, 1991; Xu et al., 1993b).

Our approach measures the contributions made by attached cross-bridges in resisting osmotic compression of the myofilament lattice. This approach is based on the observations that cross-bridges bound to actin exert forces in the radial direction that affects the separation between the thick and the thin filaments (Maughan and Godt, 1981; Matsubara et al., 1984, 1985; Millman and Irving, 1988; Schoenberg, 1980; Brenner and Yu, 1991; Xu et al., 1993b). We further found that the direction of the radial force, pushing the filaments apart or pulling them closer, and the magni-

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tude of the radial force are functions of the actual filament separation; i.e., attached cross-bridges are elastic in the radial direction (Brenner and Yu, 1991). In addition, the radial force is a function of the chemical state of the cross-bridges. At one specific filament separation, which is characteristic for each chemical state, the radial force is zero, i.e., attachment or detachment of any number of cross-bridges does not change the filament separation (Brenner and Yu, 1991; Xu et al., 1993b). This specific filament separation was defined as the "equilibrium spacing," as a way of suggesting that at this interfilament spacing attached cross-bridges are radially undistorted. Because it is the associated surface-to-surface distance between myosin and actin filaments that reflects this unique aspect of the radial elasticity rather than lattice spacing per se, we shall use the term "radial equilibrium length" of an attached cross-bridge to describe this property (equilibrium spacing and radial equilibrium length are related by Eq. 2). Our previous results indicated that the geometry (i.e., the structure) of the radially unstrained actomyosin cross-bridges is different for each nucleotide state studied so far. Thus the radial equilibrium length can be used as a unique spatial parameter that 1) allows one to screen for structural differences among attached cross-bridge states, 2) provides a quantitative estimate on the extent of the structural differences among different cross-bridge states in radial direction, and 3) quantitatively defines the radial component of the length of the actomyosin cross-bridge, which can be used as a guide/constraint for modeling the actomyosin cross-bridges into the three-dimensional filament lattice of the muscle fibers.

To determine structural changes involved in force generation, one critical step is to characterize the structural properties of the weakly attached cross-bridge states having bound ATP or ADP·P_i, in comparison with the structural properties of the strongly bound states. Thus far the radial equilibrium length of the weakly bound cross-bridges has not been determined.

In the present study the radial equilibrium lengths of three attached cross-bridge states (i.e., weakly bound, force-generating, and rigor) are determined at the same ionic strength, confirming that there is a significant structural difference between the weakly bound and the force-generating cross-bridges.

Preliminary results have been reported earlier (Xu et al., 1993a).

MATERIALS AND METHODS

Fiber preparations

Single, chemically skinned rabbit psoas muscle fibers were used throughout this study. Single fibers were prepared according to the method of Brenner (Brenner, 1983; for details, see Yu and Brenner, 1989). Muscle fiber membranes were made permeable by using a "skinning" solution that is identical to the one reported previously (Kraft et al., 1995).

All experiments were performed at 4–6°C, and at 80 mM ionic strength (μ). Sarcomere length was 2.3–2.4 μm, which was determined by laser light diffraction simultaneously with x-ray diffraction.

Solutions

Table 1 shows the composition of solutions used for various conditions. Glutathione was included to reduce radiation damage. Caffeine was included to deplete the sarcoplasmic reticulum (SR) of calcium, ensuring homogeneous activation among the sarcomeres.

The macromolecule dextran T_{500} (Pharmacia Fine Chemicals, Uppsala, Sweden) was used to apply osmotic pressure to the skinned fibers. The resultant radial force per unit length of thick filament, F_r, applied to the muscle fibers can be estimated according to the equation derived by Schoenberg (1980) and Matsubara et al. (1984):

$$F_r = 2\sqrt{3}\,\pi d_{10}, \quad (1)$$

where π is the osmotic pressure exerted by dextran T_{500}; d_{10} is the spacing between the [1,0] Bragg planes (i.e., the perpendicular distances between the planes containing the thick filaments). The concentration (w/v) of the dextran T_{500} ranged between 0% and 8%, and the corresponding osmotic pressures were assumed to be the same as those determined previously (Xu et al., 1993b). In that earlier study, no significant differences were observed among three different lots of dextran T_{500}.

Actin-binding caldesmon fragments

To determine the radial elasticity of the weakly attached cross-bridges, it was necessary to manipulate the fraction of cross-bridges weakly bound to actin without changes in ionic strength, temperature, and nucleotide conditions. It has been shown that in a relaxed skinned rabbit psoas fiber at low temperature and low ionic strength, large fractions of the cross-bridges are weakly attached to actin. To reduce the fraction of weakly bound cross-bridges in the relaxed muscle without changes in ionic strength, temperature, or nucleotide, the 20-kDa C-terminal fragment of caldesmon, which only binds to actin, was used to compete off the weakly bound cross-bridges (Brenner et al., 1991; Kraft et al., 1995). The actin-binding caldesmon fragment was prepared from turkey gizzards by a modification of the method of Bretscher et al. (Bretscher, 1984; Velaz et al., 1989, 1993). In the present study, 0.75 mg/ml of the 20-kDa caldesmon fragment was added to the relaxing solution at μ = 80 mM.

X-ray diffraction

Diffraction patterns from the rigor fibers and some of the patterns from relaxed fibers, in the presence and in the absence of caldesmon fragment, were recorded at the National Institutes of Health laboratory by using a conventional rotating-anode x-ray generator (Elliott GX-6) and a single-

TABLE 1 Composition of solutions used for various experiments in the present study

Condition	Rel 80 (preactivation)	Act 80	Rig 80	Rig 50
Imidazole (mM)	10	10	10	10
MgATP (mM)	1	1	—	—
$MgCl_2$ (mM)	2	2	—	—
EGTA (mM)	1	—	2	1
CaEGTA (mM)	—	1	—	—
EDTA (mM)	—	—	3	1
KCl (mM)	—	—	—	38
CrP (mM)	10	10	10	—
DTT (mM)	10	10	10	—
Glutathione (mM)	30	30	30	—
Caffeine (mM)	10	10	10	—
CPK (units/ml)	500	500	—	—
μ (mM)	80	80	80	50
pH	7.0	7.0	7.0	7.0

wire position-sensitive x-ray detector (Yu and Brenner, 1989). Data from fully Ca^{2+}-activated fibers under osmotic compression were obtained by using the intense synchrotron radiation source at Daresbury Laboratory (Warrington, England). Experiments were carried out at station 2.1. Diffraction patterns were recorded on a two-dimensional multiwire detector provided by the Daresbury Laboratory. The specimen-to-detector distance was 5.5 m.

Approximately one-half of the data from the relaxed fibers with and without caldesmon fragment were obtained in the National Institutes of Health laboratory and one-half were from Daresbury. No significant differences were found between the two sets of data, and the results were combined. The activated fibers presented in this paper were obtained solely from the synchrotron radiation source, whereas those of the rigor data were entirely obtained by the laboratory source.

Radial force associated with cross-bridge attachment

The radial force associated with cross-bridge attachment was estimated by following the resistance to osmotic compression applied to the muscle fibers (applied radial force), as described previously (Brenner and Yu, 1991; Xu et al., 1993b). The general concept of this technique is the following: a skinned muscle fiber is compressed by dextran T_{500} to a given filament separation under the conditions where few cross-bridges are attached. The applied radial force is proportional to the osmotic pressure generated by dextran T_{500} in the bathing solution (see Eq. 1). Subsequently, the same muscle fiber is compressed to the same filament separation under the conditions to be investigated (e.g., activated fiber, i.e., when cross-bridges are attached). The difference in the amount of applied radial force required to reach the same filament spacing is attributed to the radial force exerted by the attached cross-bridges. The compressive force (pulling the thick and the thin filaments closer) is assigned to be positive, and the expansive force (pushing the filaments apart) is assigned to be negative. In the Results, radial force is expressed in units of pN (thick filament length)$^{-1}$, with the thick filament assumed to be 1.6 μm in length.

The surface-to-surface distance, $D_{(\text{thick-thin})}$ (nm), between the thick filament backbone and the thin filament is related to the equatorial lattice spacing d_{10} of the x-ray diffraction patterns by the following expression:

$$D_{(\text{thick-thin})} = 2/3 * d_{10} - 13.0, \quad (2)$$

where the radius of the thick filament is assumed to be 8.0 nm and the thin filament is 5.0 nm.

Estimate of radial stiffness

The magnitude and direction of the radial force exerted by an attached cross-bridge generally vary with filament separation, signifying a radial elasticity. The stiffness in the radial direction is defined as the slope of radial force versus $D_{(\text{thick-thin})}$ at the point where the radial force is zero:

$$\text{Radial Stiffness} = \{\Delta(\text{radial force})/\Delta D_{(\text{thick-thin})}\}_{\text{at radial force}=0}. \quad (3)$$

The radial stiffness is expressed in units of pN (thick filament length)$^{-1}$ (nm)$^{-1}$.

Experimental protocol

Conceptually, the procedure to estimate radial forces of attached cross-bridges would be to compress the muscle fiber to a series of preassigned lattice spacings when few cross-bridges are attached and when large fractions are attached, and compare the differences in the amount of dextran used. However, it would be difficult for such a procedure to yield a series of data with a high degree of accuracy. In practice, a series of x-ray diffraction patterns from a single fiber was recorded with various concentrations of dextran T_{500} applied to the fiber. The resulting plots of $D_{(\text{thick-thin})}$ versus applied radial force are called "response curves." The response curves were fitted by an equation, generally of the form

$$G(x) = A * \exp(Bx) + C \quad (4)$$

by using MLAB (Civilized Software, Bethesda, MD). From the fitted curves, the differences in the applied radial force to reach the same lattice spacing were calculated.

For the static conditions (relaxed and rigor) the response curves were first obtained under relaxing condition in the absence of caldesmon fragment, followed by rigor condition, or relaxing condition with caldesmon fragment.

In studying activated fibers, we recorded the diffraction patterns of relaxed muscle in the preactivating solution with dextran T_{500} before the patterns from activated fibers at the same concentration of dextran T_{500}. The response curves for the relaxed fibers in the presence of caldesmon (i.e., few cross-bridges are attached) were obtained from a batch of fibers separate from the active fibers, since it was found to be difficult to maintain good lattice order to take data from the same fiber under these two conditions.

RESULTS

Previously the characteristics of radial force and radial elasticity were determined under conditions where the cross-bridges were strongly attached to actin, e.g., in active contraction, in rigor, and in the presence of MgADP or $MgPP_i$ at an ionic strength of 170 mM. Since in the present study we intended to determine the radial properties of weakly attached cross-bridges, a reduced ionic strength of 80 mM was used to increase the fraction of weakly attached cross-bridges.

Ionic strength has large effects on the lattice spacing (Brenner et al., 1984). To avoid complications due to different charge distributions on the filaments, we also examined the radial mechanical properties of the fully Ca^{2+}-activated and rigor fibers under the same ionic strength of 80 mM used for relaxed fibers.

Response of filament lattice to osmotic compression when weak cross-bridge attachment is reduced substantially

We determined the response of the filament lattice to osmotic compression under relaxing conditions (μ = 80 mM) with as few weakly attached cross-bridges as possible. To achieve this, the 20-kDa fragment of caldesmon was added to a relaxing solution (0.75 mg/ml final) to inhibit weak cross-bridge attachment (Brenner et al., 1991; Kraft et al., 1995). At this concentration of caldesmon fragment, the relaxed fiber stiffness was reduced by approximately 75% at μ = 50 mM (figure 4 in Brenner et al., 1991) and by 45% at μ = 170 mM (Kraft et al., 1995). It is reasonable to assume that inhibition at μ = 80 mM is approximately 65%. Meanwhile, at μ = 80 mM in the absence of caldesmon, the relaxed fiber stiffness is about 50% of that at μ = 20 mM (figure 3 in Kraft et al., 1995), i.e., less than 50% of the

cross-bridges could be weakly bound to actin under this condition. Therefore, in the presence of 0.75 mg/ml of caldesmon fragment, approximately 10–15% of cross-bridges are weakly attached.

Changes in $D_{(thick\text{-}thin)}$ as a result of applied radial force are shown in Fig. 1. This response curve serves as a reference for determining radial forces exerted by the attached cross-bridges in the absence of caldesmon fragment under relaxing conditions (weakly bound cross-bridges), active contraction (force generating cross-bridges), and rigor.

In the presence of 0.75 mg/ml of caldesmon fragment, the equatorial intensities showed a high degree of disorder of lattice structure when the dextran concentration exceeded 3% (w/v), possibly due to the fact that few of the cross-bridges are attached to stabilize the thin filament at the trigonal point of the hexagonal filament lattice. Because of this disorder, the data shown in Fig. 1 are limited to 3% of dextran T_{500} (applied radial force = 219 pN (thick filament length)$^{-1}$).

Radial force resulting from weak cross-bridge binding in relaxed muscle fibers

From the same muscle fibers, the response to osmotic compression was also recorded under relaxing conditions in the absence of the caldesmon fragment (Fig. 2, *filled circle*). When the osmotic compression is zero, $D_{(thick\text{-}thin)}$ in this case is reduced from 13.0 nm ± 0.03 nm (SEM) to 12.7 nm ± 0.03 nm (SEM) upon the removal of caldesmon fragment (i.e., upon additional weak cross-bridge attachment). This is consistent with the concept that there is a radial force associated with the weak cross-bridge binding, and the force pulls the filaments closer (positive direction). When the fibers are precompressed to $D_{(thick\text{-}thin)} < 12.0$ nm, the lattice spacing increases upon removal of caldesmon. This is consistent with the idea that below 12.0 nm the radial force of the weakly bound cross-bridges pushes the filament lattice apart (i.e., turns to negative direction). However, at $D_{(thick\text{-}thin)} = 12.0$ nm, there is no spacing change upon the removal of caldesmon fragment, suggesting that at this interfilament distance, weakly bound cross-bridges do not exert any radial force. Therefore the radial equilibrium length of the weakly bound cross-bridges is 12.0 nm.

Radial forces exerted by force-generating cross-bridges and by cross-bridges in rigor

When a fiber was fully activated by calcium in the absence of dextran T_{500}, $D_{(thick\text{-}thin)}$ was 11.7 nm (Fig. 3). When this is compared with the $D_{(thick\text{-}thin)}$ of the relaxed fiber, it is smaller. Thus in the absence of dextran, cross-bridges in the activated fibers tend to pull the filaments closer. Comparison of the response curves from activated fibers and relaxed fibers in the presence of caldesmon fragment shows that the two curves converge approximately at a $D_{(thick\text{-}thin)}$ of 10 nm. Because of the limited range of the response curve in the presence of caldesmon fragment, the two response curves do not cross each other. However, if one uses the response curve c (thin solid line in Fig. 3), the two curves cross each other at $D_{(thick\text{-}thin)} = 10.3$ nm. Since the re-

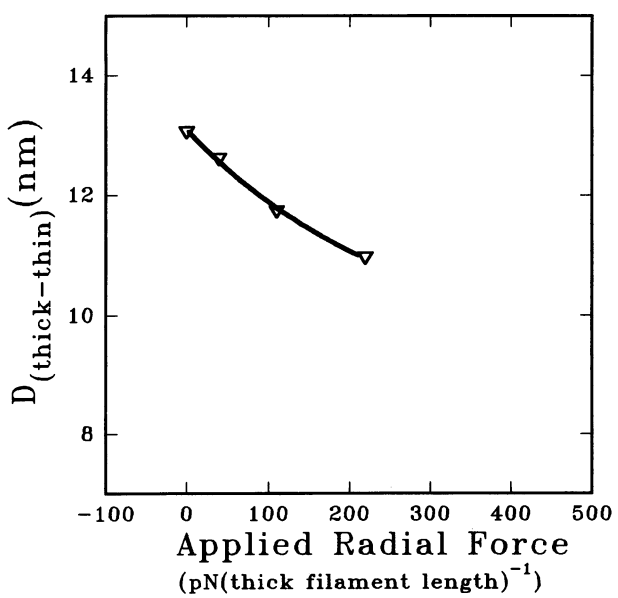

FIGURE 1 Response curve (surface-to-surface distance $D_{(thick\text{-}thin)}$ as a function of applied radial force) of a relaxed fiber in the presence of 0.75 mg/ml of 20-kDa actin-binding caldesmon fragment. μ = 80 mM. ▽, Actual data. The solid line was obtained by a least-squares fit to $G(x) = a * \exp(bx) + c$, where $a = 37.33$, $b = -0.0627$, $c = 210.73$. Standard errors of the mean (SEMs) are smaller than the size of the symbols.

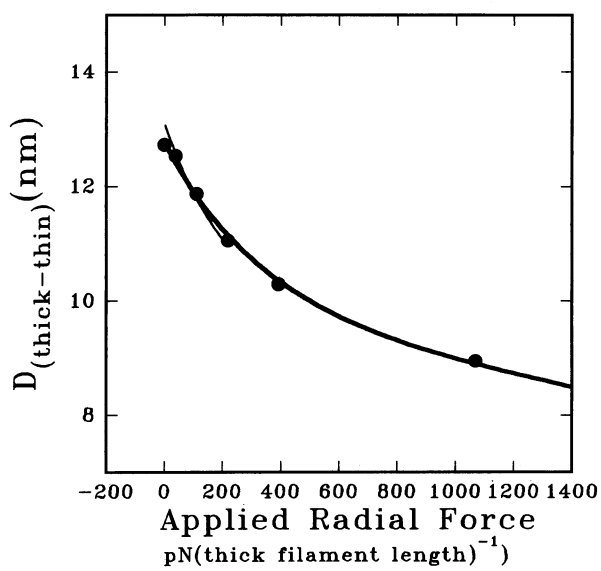

FIGURE 2 Response curve of relaxed fibers in the preactivating solution (Table 1) in the absence of the caldesmon fragment (●). The experimental conditions were otherwise the same as in Fig. 1. The thick solid line was fitted to $F(x) = a * \exp(bx) + c * \exp(dx) + e$, with $a = 26.54$, $b = -0.0506$, $c = 217.73$, $d = -0.000849$, and $e = 0.747$. The thin solid line is the fitted response curve shown in Fig. 1. SEMs are smaller than the diameter of the symbols.

sponse curve c of the relaxed fiber is shifted right compared to curve a, the cross-over between curves a and b is expected at a $D_{(\text{thick-thin})}$ somewhat larger than 10.3 nm. Extrapolation of the fitted curve a yields a radial equilibrium length of 10.5 nm. Therefore, it is reasonable to conclude that at μ = 80 mM the radial equilibrium length of the force-generating cross-bridges is ~10.5 nm.

For the fibers under the rigor condition without osmotic compression, the $D_{(\text{thick-thin})}$ (13.0 nm) was the same as that of relaxed fiber in the presence of caldesmon (Fig. 4). As the concentration of dextran T_{500} was increased, rigor attachment pushed the filaments apart; i.e., the radial force in the positive (pulling) direction was not observed. However, as was reported previously (Brenner and Yu, 1991; Xu et al., 1993b), at higher ionic strength (170 mM) where the filament separation under relaxing condition is greater, rigor attachment exhibited radial force in both directions and the radial equilibrium length was found to be 12.2 nm (Brenner and Yu, 1991). Under the present condition (μ = 80 mM), the radial equilibrium length of rigor attachment appears to be shifted to 13.0 nm and the radial force in the positive direction, by coincidence, may not be observable.

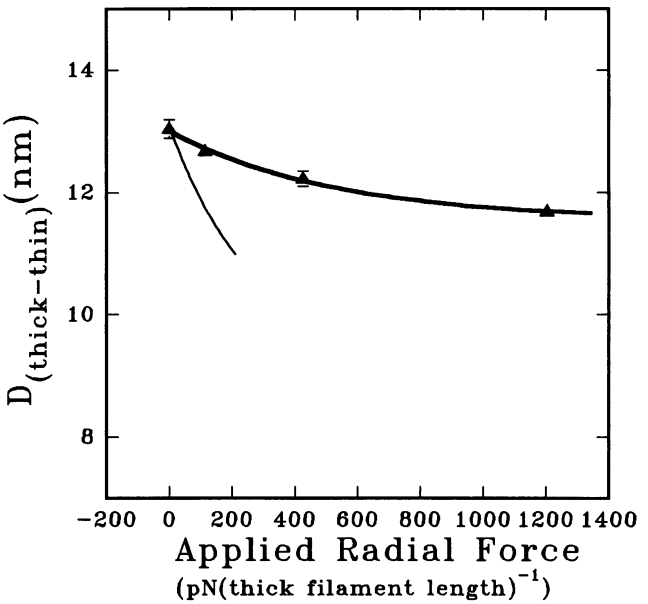

FIGURE 4 Response curve of rigor fiber (▲). The thick solid curve was fitted to $G(x) = a * \exp(bx) + c$, with a = 14.63, b = −0.0307, and c = 232.5. The thin solid line is the fitted response curve of the relaxed fiber in the presence of caldesmon fragment, as shown in Fig. 1.

Estimated radial stiffness of the attached cross-bridges

Radial forces exerted by the attached cross-bridges are functions of the distance between the filaments, as shown in Fig. 5; i.e., the cross-links are elastic in the radial direction. The resulting radial stiffness of the attached cross-bridges can be determined as the slope of the curves in Fig. 5 at the points where radial force is zero. The radial stiffness is 31.2 pN (single filament)$^{-1}$ (nm)$^{-1}$ for relaxed fibers with cross-bridges weakly attached to actin (Fig. 5 *b*); 151.2 pN (single filament)$^{-1}$ (nm)$^{-1}$ for the activated fibers (Fig. 5 *a*); and 295.2 pN (single filament)$^{-1}$ (nm)$^{-1}$ for fibers in rigor (Fig. 5 *c*).

The radial stiffness is derived with the assumption that the elasticity resides only in the myosin heads with the thin filament and the backbone of the thick filament is rigid. However, if the radial force exerted by the attached cross-bridges causes some bending (zigzag) in the filaments, the radial cross-bridge stiffness is less than that estimated here.

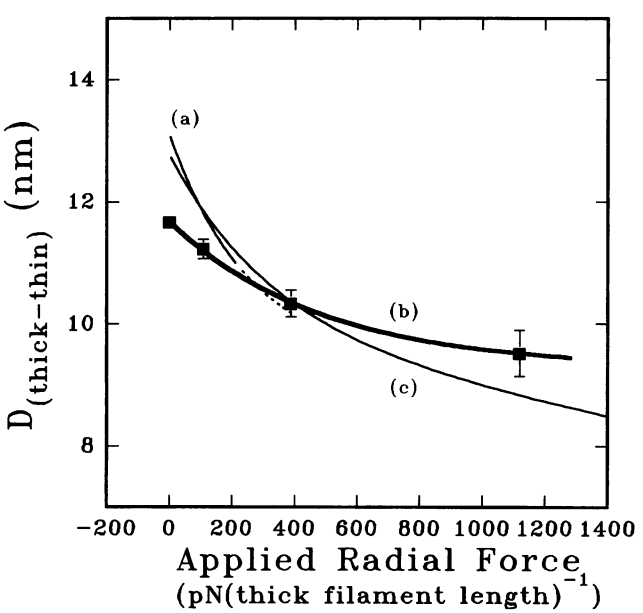

FIGURE 3 Response curve of fully Ca^{2+}-activated fiber (■). Experimental conditions were otherwise the same as in Fig. 2. The thick solid line (b) was fitted to $G(x) = a * \exp(bx) + c$, where a = 23.95, b = −0.0332, and c = 209.84. Lines a and c are the fitted curves shown in Fig. 2 for the relaxed fiber in the presence and in the absence of caldesmon fragment.

Control experiment: binding of caldesmon does not affect the muscle fiber's response to osmotic pressure

It could be argued that binding of caldesmon per se could affect the equilibrium spacings and/or the radial stiffness. To test such a possibility, caldesmon was added to rigor fibers. It has been shown that caldesmon is not an effective competitive inhibitor for strongly bound cross-bridges. In a muscle fiber there are approximately 50% unoccupied actin sites on the thin filament, even if all of the myosin heads are bound to actin. Therefore, some caldesmon fragments are expected to bind to actin even under the rigor conditions. To promote stronger binding of the 20-kDa caldesmon fragment to actin, the control experiment was performed at μ = 50 mM. In the presence of 0.2 mg/ml of caldesmon fragment, the equatorial intensity ratio I_{11}/I_{10} increased by 11%,

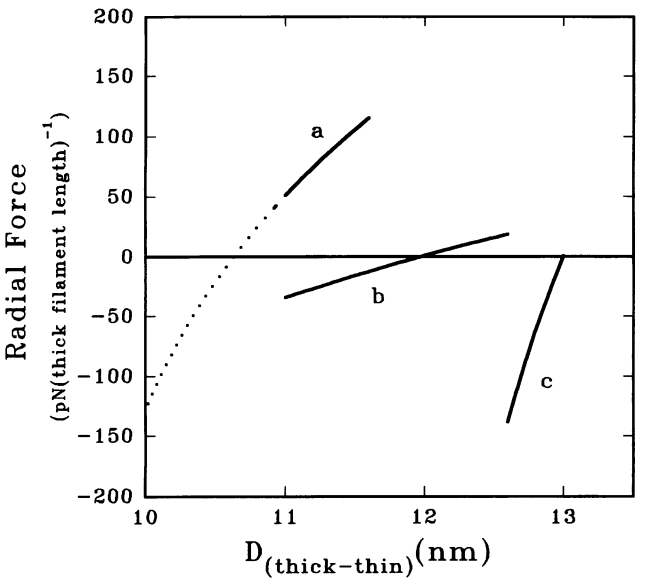

FIGURE 5 Radial forces generated by attached cross-bridges in (*a*) Ca^{2+}-activated force generating state, (*b*) weakly bound states in relaxed fibers, and (*c*) in rigor state. Curve a was calculated by subtracting the curve fitted for the activated state (Fig. 3) from that of the relaxed fiber in the presence of caldesmon fragment at the same surface-to-surface distance. The dotted line is based on the extrapolated curve for the relaxed fiber in caldesmon shown in Fig. 3. The active radial force is extrapolated to reach zero at $D_{(thick-thin)}$ = 10.5 nm. Similar calculations were made for the weakly bound cross-bridges (*b*) and the cross-bridges in rigor (*c*). The radial forces reach zero at $D_{(thick-thin)}$ = 12.0 nm and $D_{(thick-thin)}$ = 13.0 nm, respectively, for *b* and *c*.

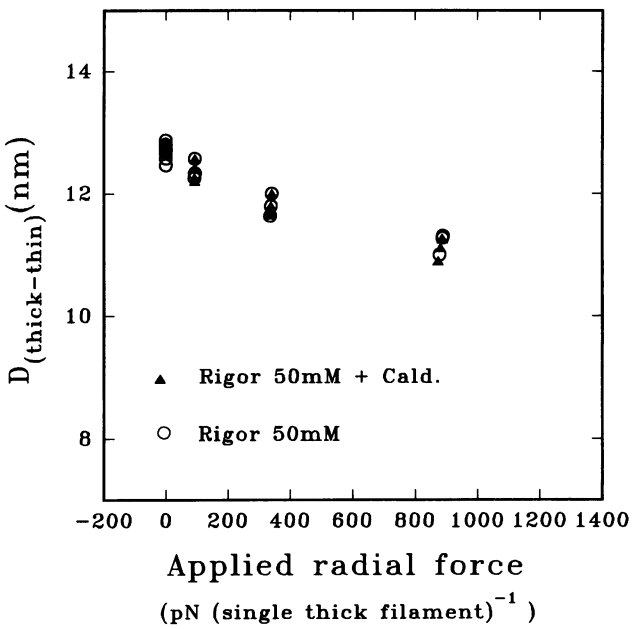

FIGURE 6 Response curves obtained from fibers in rigor in the presence (▲) and in the absence (○) of 0.2 mg/ml caldesmon fragment. An ionic strength of 50 mM was used to promote stronger binding of caldesmon fragment to actin. The response of the fiber to osmotic pressure is clearly not affected by the binding of the caldesmon fragment.

suggesting some binding of caldesmon fragment to the actin filament at sites not occupied by bound myosin heads. The response to osmotic pressure is not affected by the bound caldesmon fragment (Fig. 6).

DISCUSSIONS

Radial mechanical properties of the weakly bound cross-bridges in relaxed muscle fibers

In our previous studies, radial mechanical properties were characterized only for cross-bridges in various strongly bound states. Thus far, evidence for a radial elasticity associated with weakly bound cross-bridges has been inconclusive. Characterization of the radial elasticity by the osmotic technique requires a comparison of compressibility of fibers with a small versus a large number of attached cross-bridges under otherwise identical conditions, e.g., at the same ionic strength. Yet methods for manipulating the fraction of weakly bound cross-bridges while keeping the ionic strength constant were not available until the selective inhibitory property of caldesmon was demonstrated recently (Brenner et al., 1991; Kraft et al., 1995).

The present results clearly demonstrate for the first time that in spite of their low affinity for actin, cross-bridges that are weakly attached to actin do exert radial force both in the compressive and in the expansive directions and the radial force is a function of the distance between filaments. This further generalizes our earlier findings that all attached cross-bridges exhibit nearly linear elastic properties in the radial direction.

Distinct radial equilibrium lengths under the three conditions studied

The three radial equilibrium lengths of 12.0 nm, 10.5 nm, and 13.0 nm most likely signify different attachment modes or different molecular structures. The present results further support our earlier findings that there is a structural change upon activation (Brenner and Yu, 1993). Although our data do not provide information on the origin of the differences in the radial equilibrium lengths, it is notable that the radial equilibrium length for the activated fibers is smaller than in the other two states. It is possible that a substantial shape change in the myosin head takes place. It is also likely that there is a change in the angle of attachment with an axial and/or azimuthal component.

Values of the radial equilibrium length obtained under the present condition may differ from those under physiological conditions, particularly for the weakly bound cross-bridges, because at higher temperatures (e.g., 20°C) the lattice spacing d_{10} of relaxed muscle fibers is reduced (from 400 Å to 380 Å, at μ = 50 mM) without osmotic compression (Xu et al., manuscript in preparation). However, when the ionic strength is changed from 80 mM to 170 mM, the radial equilibrium lengths for the activated and the rigor cross-bridges remain significantly different (see Brenner and Yu,

1991). We believe that the exact values of the radial equilibrium length may differ under physiological conditions, but significant differences among the three conditions should remain.

Reliability of the values of equilibrium spacings

In the present study the actin-binding caldesmon fragment was used to reduce the fraction of attached cross-bridges. Our control experiment showed that the binding of caldesmon per se does not appear to affect the equilibrium spacings.

Radial equilibrium length is derived by determining the point where the response curves cross each other. This cross-over point was determined by the intersection of two curves fitted to the experimental data, making it difficult to estimate the statistical errors in the estimate. However, if curves were fitted to the mean values of $D_{(\text{thick-thin})} \pm$ SEM by the same type of equations used for fitting their mean $D_{(\text{thick-thin})}$ values and differences were then taken between the curves thus obtained (for details, see (Brenner and Yu, 1991; Xu et al., 1993b), the estimated radial equilibrium lengths remain distinct from each other for the three conditions studied and therefore the differences are statistically significant.

It might be argued that a certain fraction, albeit small (~10-15%), of cross-bridges remains attached to actin in the presence of 0.75 mg/ml of actin-binding caldesmon fragment. This remaining fraction might affect the determination of the radial equilibrium length, because the response curve with no attachment would be steeper (but still crossing the response curve (Fig. 3 *c*) at 12 nm). However, the effect is likely to be very small, since the radial force exerted by the weakly bound cross-bridges is very low. For instance, the radial equilibrium length for the fully activated fiber at μ = 80 mM and 4°C would be shifted by 0.4 nm to 10.2 nm if it were obtained by simply comparing the radial force originating from the relaxed fiber (with ~50% of cross-bridges weakly attached) with that from the activated fiber. Even if we assume that 25% of the cross-bridges are weakly attached in the presence of caldesmon fragment, the error in estimating the equilibrium spacing will be smaller than 0.4 nm. This would raise the radial equilibrium length to less than 11 nm, still significantly distinct from those for weakly bound and rigor cross-bridges.

Relevance of weak cross-bridge attachment for the stability of the filament lattice

By virtue of their radial elasticity, the weakly attached cross-bridges may play a significant role in stabilizing the filament lattice of the relaxed muscle fibers. When few cross-bridges remain attached in the presence of high concentrations (e.g., >0.4 mg/ml at μ = 50 mM and 4°C) of the 20-kDa caldesmon fragment, the [1,1] reflection in the equatorial x-ray diffraction patterns becomes broad and barely visible above the background, signifying a high degree of disorder in the position of the thin filaments (Malinchik and Yu, 1995). Previously, Matsuda and Podolsky showed that strongly bound cross-bridges in rigor kept the thin filaments at the trigonal points of the filament lattice under low pH conditions (Matsuda and Podolsky, 1986; Podolsky et al., 1991). Combining the two results, it appears that cross-bridge attachment, weak or strong, is critical in stabilizing the trigonal position of the thin filaments in the A-band of the muscle fibers.

Significance for modeling actomyosin interactions in muscle: unique spatial constraints

The present finding further supports our earlier suggestion that radial equilibrium lengths of the attached cross-bridges depend on the nucleotide state. It follows that the conformation of the cross-link (e.g., the molecular structure of the myosin or the orientation of attachment) varies with the nucleotide state.

The distinct equilibrium lengths not only indicate structural differences between various nucleotide states, but also provide unique spatial constraints for modeling actomyosin interaction in the three-dimensional filament space. Unlike the density envelope provided by the electron microscopy of S1 decorated actin filament, the present findings provide spatial constraints for deformed myosin heads (cross-bridges) in the filament lattice. Experimental results have indicated that there is only one radial equilibrium length associated with each cross-bridge state. Furthermore, the value of the equilibrium length does not depend on the number of cross-bridges attached. These are the unique features that render special significance to the concept of radial equilibrium length. In modeling the actomyosin interaction in muscle, the modeled structure must be consistent with the spatial constraint available for each state.

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