Backward Movements of Cross-Bridges by Application of Stretch and by Binding of MgADP to Skeletal Muscle Fibers in the Rigor State as Studied by X-Ray Diffraction

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ABSTRACT The effects of the applied stretch and MgADP binding on the structure of the actomyosin cross-bridges in rabbit and/or frog skeletal muscle fibers in the rigor state have been investigated with improved resolution by x-ray diffraction using synchrotron radiation. The results showed a remarkable structural similarity between cross-bridge states induced by stretch and MgADP binding. The intensities of the 14.4- and 7.2-nm meridional reflections increased by ~23 and 47%, respectively, when 1 mM MgADP was added to the rigor rabbit muscle fibers in the presence of ATP-depletion backup system and an inhibitor for muscle adenylate kinase or by ~33 and 17%, respectively, when rigor frog muscle was stretched by ~4.5% of the initial muscle length. In addition, both MgADP binding and stretch induced a small but genuine intensity decrease in the region close to the meridian of the 5.9-nm layer line while retaining the intensity profile of its outer portion. No appreciable influence was observed in the intensities of the higher order meridional reflections of the 14.4-nm repeat and the other actin-based reflections as well as the equatorial reflections, indicating a lack of detachment of cross-bridges in both cases. The changes in the axial spacings of the actin-based and the 14.4-nm-based reflections were observed and associated with the tension change. These results indicate that stretch and ADP binding mediate similar structural changes, being in the correct direction to those expected for that the conformational changes are induced in the outer portion distant from the catalytic domain of attached cross-bridges. Modeling of conformational changes of the attached myosin head suggested a small but significant movement (about 10–20°) in the light chain-binding domain of the head toward the M-line of the sarcomere. Both chemical (ADP binding) and mechanical (stretch) intervensions can reverse the contractile cycle by causing a backward movement of this domain of attached myosin heads in the rigor state.

INTRODUCTION

Active force of muscle is thought to be generated by a change in the conformation or a movement of an actin-myosin head complex (cross-bridge). This leads to the possibility that a passive force (external stretch) could in turn alter cross-bridge configuration and produce a backward movement. Naylor and Podolsky (1981) first investigated the effect of stretch application on rigor cross-bridge configuration by x-ray diffraction and found no apparent changes in the equatorial diffraction. Later, their conclusion was supported by Cooke (1981) and Hambly et al. (1991) who found little effect of external loads on the orientation of spin probes attached either to the catalytic domain (a reactive thiol, SH1 (Cys707)) or a regulatory light chain sitting on the tail domain of the myosin heads in rigor muscle. Recently, Burghardt et al. (1997) also found that fluorescent probe bound to a Cys707 did not rotate when rigor muscle fibers were rapidly stretched. However, Tanaka et al. (1991) and recently Yagi et al. (1996) reported by x-ray diffraction that the 14.4-nm meridional intensity changed in response to tension changes when slow and fast sinusoidal length changes were applied to rigor muscles, respectively. Recently, Irving et al. (1995) observed a small but significant change in the orientation of fluorescent dyes attached to the regulatory light chain in a myosin when the rigor muscle fiber was rapidly stretched, suggesting that the cross-bridge changes are confined to that region.

It is well known that MgADP binds with high affinity to myosin heads in glycerinated muscles and promotes the formation of an actin-myosin-ADP ternary complex, lowering rigor tension (Marston, 1973; Marston et al., 1976; Schoenberg and Eisenberg, 1987; Dantzig et al., 1991; Tanner et al., 1992). Rodger and Tregear (1974) investigated the x-ray diffraction patterns from the permealized insect flight muscle in rigor in the presence of MgADP, and concluded that the conformation of the attached heads was not altered by the binding of ADP. X-ray and birefringence studies with skeletal muscle fibers also showed that MgADP had no apparent structural effect on the attached myosin heads (Obiorah and Irving, 1989). However, electron paramagnetic resonance (EPR) studies on spin probes attached to a Cys707 of the heads showed a small angle change of the probe in the presence of MgADP (Ajtai et al.,

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1989; Fajer et al., 1990). A recent fluorescent polarization study revealed a small but distinct angle change of the probe attached to the regulatory light chain in a myosin upon photo-producing ADP in the rigor rabbit fibers (Allen et al., 1996). EPR studies on the spin probes attached to a light chain failed to detect such an angle change (Arata, 1990; Hambly et al., 1991; Gollub et al., 1996). Very recently, three-dimensional reconstruction of electron micrographs of the actin filaments decorated with S1 (subfragment-1) from smooth muscle myosin revealed that an ADP binding induced a considerable degree of movement or rotation of a light chain-binding domain of the attached heads (Whittaker et al., 1995). EPR spectroscopy also detected a similar degree of ADP-induced angle change of the spin probe attached to the regulatory light chain of smooth muscle S1 bound to the actin filaments in rigor muscle fibers (Gollub et al., 1996). But the similar changes have not been seen using skeletal muscle S1 (Gollub et al., 1996; Barsotti et al., 1996; Jontes et al., 1997). However, earlier electron microscopy (EM) studies on myosin molecules using a shadowing technique revealed that a population of bent-formed myosin heads increased in the presence of ADP (Tokunaga et al., 1991). Recent x-ray solution scattering have shown that a distinct compaction of the skeletal muscle S1 shape occurs in the presence of MgADP (Sugimoto et al., 1995, 1996; Sugimoto, Tokunaga and Wakabayashi, submitted for publication). Thus, there has been much controversy as regards the effects of an ADP binding and external loads on the rigor cross-bridges in a skeletal muscle.

We have reinvestigated in details the effects of the application of stretch and the binding of MgADP to the myosin heads attached to thin actin filaments in the rigor state of skeletal muscle fibers using improved x-ray diffraction techniques with a high-sensitive area detector and intense synchrotron x-rays. We found that upon the application of stretch significant intensity changes of the 14.4-nm-based meridional reflections occurred with an accompanying of a small but genuine intensity change of the 5.9-nm actin-based layer line. Interestingly, these changes closely resembled those brought about upon the addition of MgADP to rigor muscle. These coordinated intensity changes indicate that stretch and ADP binding seem to mediate similar structural changes in the light chain-binding region distant from the catalytic domain and from actin. Preliminary results were reported in the abstract form (Takezawa et al., 1996; Kim et al., 1996).

MATERIALS AND METHODS

Specimen preparation and mechanical experiments

For the ADP binding experiments, freshly glycerinated rabbit psoas muscles were prepared by a conventional method (e.g., Tawada and Kimura, 1984). The specimens for x-ray diffraction experiments consisted of a bundle of 10–15 single fibers. The sarcomere length of the fiber bundle was adjusted to ~2.4 μm using optical diffraction with a He-Ne laser. Such a bundle was mounted in a Perspex chamber with two Mylar windows for x rays to pass through. After washing out glycerination solution, rigor solution (80 mM K-propionate, 5 mM EGTA, 6 mM Mg-$(acetate)_2$, and 40 mM imidazole/HCl buffer (pH 7.0)) was circulated through the chamber. For addressing the effect of ADP binding, an inhibitor for muscle adenylate kinase (0.2 mM diadenosine pentaphosphate (AP_5A)) and the ATP-depletion backup system (0.1 mg/ml or ~3 units/ml hexokinase-1 mM glucose) were added to the solution to prevent the build-up of ATP in a fiber and to decompose contaminant ATP in ADP solution, respectively. The use of the backup system and AP_5A was absolutely necessary for investigating the ADP binding effects.

Whole sartorius muscles of the small bullfrog (*Rana castesbeiana*) were used for the stretch experiments. Intact frog muscles were rigorized at the full filament overlap in cold Ringer solution (4°C) (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 5 mM NaH_2CO_3 (pH 7.2)) containing 2 mM iodoacetic acid for >36 hr as described previously (Huxley and Brown, 1967). The pelvic end of the muscle was clamped to the hook in the chamber whereas the tibial end or tendon was connected to a force transducer.

Stretch experiments were performed as follows. When rigor muscle was held isometrically and any slackness in the muscle was taken up, rectangular length changes (~4.5% of the initial muscle length (L_0) with a velocity of ~0.4 L_0/s and a duration of 4.5 s) were applied using a moving coil driver controlled with a feed-back circuit. The average tension at a 4.5%-L_0 stretch corresponded to ~0.5 P_o in which P_o denotes the maximum isometric tension (2.5 ± 0.2 kg/cm^2), which was measured in a separate tetanic experiment using the intact preparation from the another leg. In the present experiments, the muscle was stretched by a force that never exceeded ~50% of isometric tension so as to avoid "give" or any damage.

X-ray diffraction experiments

X-ray experiments were carried out at the Beamline 15A at the Photon Factory (Tsukuba, Japan) using synchrotron radiation from a positron storage ring. Monochromatic x-ray beam (wavelength, $\lambda = 0.1507$ nm) was selected and collimated by using doubly focusing optics (Amemiya et al., 1983; Wakabayashi and Amemiya, 1991). The storage ring was operated (in a medium-ϵ optics) at 2.5 GeV with a beam current between 300 and 350 mA. The x-ray diffraction patterns were recorded on a storage phosphor area detector (an imaging plate (type BAS-III), Fuji Film, Tokyo, Japan) (e.g., Amemiya et al., 1987) in a very short exposure time.

For the ADP binding experiments, the fiber bundle was set horizontally so as to make the optimum use of collimated x-ray beam. The size of the x-ray beam was 2.3 (H) × 0.4 (V) mm^2 at the specimen. Three diffraction patterns were recorded from the same specimen sequentially to show up to the 2.7-nm actin-based meridional reflection at the specimen-to-plate distance of 120 cm in a total exposure time of 30–45 s. The first record was taken from the specimen in rigor solution, the second one from the specimen in the solution containing MgADP, and the last one from the specimen returned to rigor solution to ensure a recovery of the original pattern. The position of the specimen where x-rays hit was varied at every exposure. No appreciable radiation damage to the specimen was observed after a total exposure of less than 3 min. The temperature was maintained at 20°C. Before each x-ray exposure, the specimen was incubated in each perfusion solution for at least 20 min. Solution was continuously circulated through the specimen chamber during the exposure by a peristaltic pump.

For experiments in which stretches were applied to a whole rigor muscle of frog, the muscle was set vertically. The horizontal beam size was widened out into ~4 mm at the specimen. Three diffraction patterns were recorded for 3 s before, during, and after stretch with the specimen-to-plate distance of 160 cm at 10°C. During stretch, the tension was recorded with a digital oscilloscope (Nicolet 3091, Madison, Wisconsin), and the x-ray shutter was opened for 3 s after an initial fast decay of tension. Alternate stretching and releasing were imposed five times for each muscle at intervals of 12 s, so that x-ray signals could be summed in a total exposure time of 15 s. During an x-ray exposure, the chamber was perfused with Ringer solution.

In both experiments, the imaging plate after the x-ray exposure was scanned by an image plate reader (BAS 2000, Fuji Film, Tokyo, Japan) using a pixel size of 100 μm. To minimize the variation in the intensity measurements, the same plate was used in a series of all experiments, and the time interval between the exposure and scanning was kept constant (for the fading effect of an imaging plate, see Amemiya, 1995).

Intensity measurements and data analysis

Digital intensity data from the image plates were analyzed on graphics workstations (Spark Station 330, Sun Microsystems, Palo Alto, California and Power Macintosh 7200, Apple Computer Inc., Cupertino, California). After determining the origin of the images and correcting the inclination angle of each image, the four quadrants of the patterns were folded and averaged. Scaled by the total intensity of the pattern (except for the strong equatorial region and around the beam stop), the data from three or more muscles were summed to increase the signal-to-noise ratio. The intensity distributions of the layer lines were measured by scanning the data along rows of pixels perpendicular to the layer lines. The background line under each peak was estimated by drawing a smooth curve connecting minimum points on each side of the peak. The integrated intensities of these peaks were then obtained by summing up the data in the area above the background, and plotted as a function of the reciprocal radial coordinate, R ($= 2\sin\theta/\lambda$ in which θ and λ are half the scattering angle and x-ray wavelength used, respectively). In order to directly obtain the integrated intensities of the layer-line reflections as well as the meridional reflections, those of their main peaks were measured in the axial direction by radial integration, on stripes parallel to the meridian in appropriate radial ranges, and the background intensity was subtracted as above.

Spacing measurements were made as described previously (Wakabayashi et al., 1994). For a meridional reflection at $\sim$1/2.7 nm^{-1} and the two layer-line reflections at $\sim$1/5.1 nm^{-1}, and $\sim$1/5.9 nm^{-1}, the intensity measurements were made in the reciprocal range of $0 \leq R \leq 0.018$ nm^{-1} (in which the layer lines appeared with a least fanning effect) by radial integration. The centroid of each axial reflection profile was determined by fitting a Gaussian to that profile. The intensities of the meridional reflections such as the first to the fifth orders of the 14.4-nm repeat were measured in the radial range of $0 \leq R \leq 0.022$ nm^{-1} where their main peak-profiles were included, and their centroids were determined as above. In the case that the reflections partially overlapped neighboring reflections, the raw data points were fitted to a model consisting of two or more overlapping Gaussian peaks using a nonlinear least squares method.

RESULTS

Similarity between structural changes of the cross-bridges induced by the binding of MgADP and by the application of stretch

Fig. 1 *A* shows a pair of x-ray diffraction patterns from rabbit muscle fibers in the rigor state in the absence (left half) and presence of MgADP (right half). The x-ray pattern from the fibers in the presence of MgADP was very similar to the original rigor pattern as was observed previously in insect flight muscle fibers by Rodger and Tregear (1974). Fig. 1 *B* shows a pair of x-ray patterns from rigor frog muscle fibers (left half) and upon applying the stretch of $\sim$4.5% initial muscle length (L_0) to the muscle (right half). Again, the effect of such an external stretch was not so obvious. Upon closer examination, there were small but significant intensity changes in both cases.

Fig. 2 shows the intensity traces of the equator. In the presence of MgADP (Fig. 2 *A*), the intensities of the 10 and

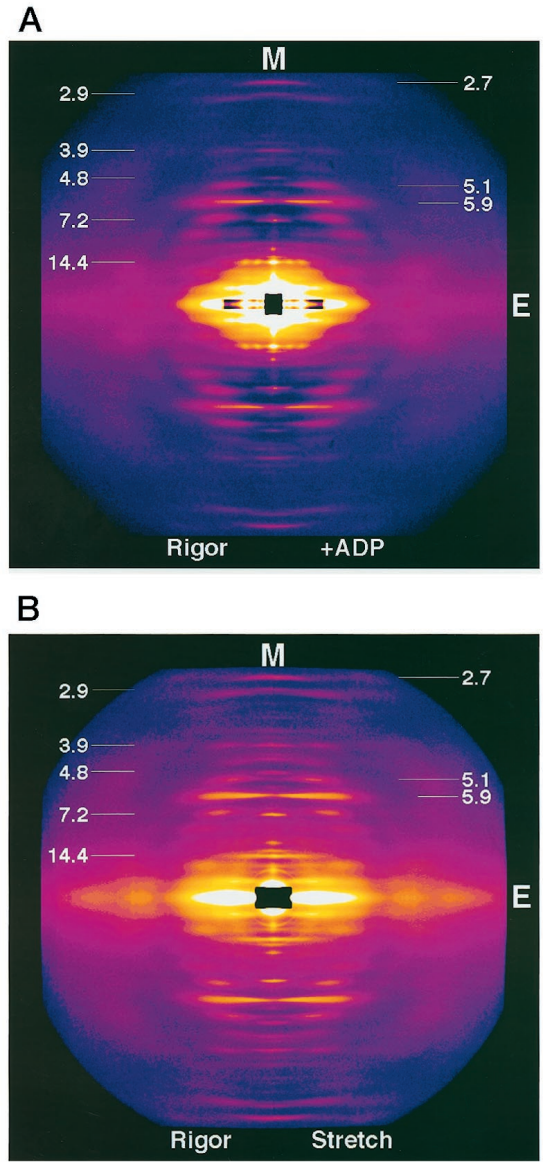

FIGURE 1 X-ray diffraction patterns from rabbit and frog skeletal muscle fibers in the rigor state in the presence of MgADP (*A*) and by the application of stretch (*B*). (*A*) The left half shows the pattern obtained from rabbit psoas fibers in the absence of nucleotide and the right half shows that obtained in the presence of 1 mM MgADP. Solutions contained 0.1 mg/ml hexokinase, 1 mM glucose, and 0.2 mM diadenosine pentaphosphate. X-ray patterns were taken in 30-s exposure. (*B*) The left half shows the pattern obtained from frog sartorius whole muscle at rest length in the rigor state. The right half shows that obtained upon the application of stretch of $\sim$4.5% of the initial muscle length (L_0) (the tension corresponding to $\sim$50% of the maximum active tension (P_0)). X-ray patterns were taken in 15-s exposure. In *A* and *B*, the appropriate Gaussian-form background intensity was subtracted for an illustration purpose. The fiber axis is vertical; M, meridional axis; E, equatorial axis. The meridional reflections of the 14.4-nm repeat and the reflection at $\sim$1/3.9 nm^{-1} (the eleventh order of the 43-nm basic repeat of the thick myosin filament) are marked on the left-hand side, and the 2.7-nm meridional and 5.1- and 5.9-nm actin-based layer-line reflections are marked on the right-hand side.

11 Bragg reflections remained mostly unchanged, although they showed a tendency of small decrease (within $\sim$5%). However, the peak positions tended to shift toward the

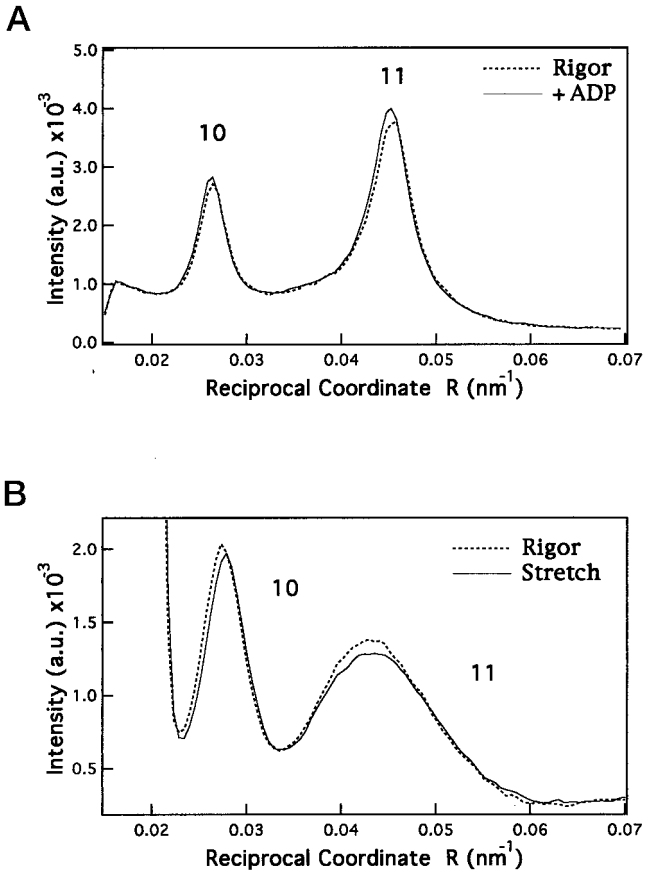

FIGURE 2 The intensity traces of the 10 and 11 equatorial reflections. (*A*) Comparison of the traces from rabbit rigor muscle fibers in the absence (*chain curve*) and presence of MgADP (*solid curve*). (*B*) Comparison of those from frog rigor muscle before (*chain curve*) and during the 4.5%-L_0 stretch (*solid curve*). *R* denotes the reciprocal radial coordinate.

low-angle side, suggesting a slight lattice swelling (~1%). This lattice expansion may be related to a slight suppression of rigor tension that was observed upon the binding of MgADP to the cross-bridges and/or caused by an ionic change in solution. In agreement with the previous results (Naylor and Podolsky, 1981; Podolsky et al., 1982), the application of stretch caused a slight lattice shrinkage but no appreciable changes (within ~5%) in these equatorial intensities (Fig. 2 *B*), indicating no apparent detachment of cross-bridges during stretch.

The most appreciable and common intensity changes were observed in the meridional reflections indexed to the 14.4-nm repeat in both cases. Fig. 3 shows the changes in the intensity distributions of the first (14.4 nm) and second (7.2 nm) order reflections of this repeat along their layer lines. The 14.4- and 7.2-nm reflections in the radial range of $0 \leq R \leq 0.040$ nm^{-1} were intensified by ~23 and 47%, respectively, in the presence of MgADP (Fig. 3 *A*). The observed intensity changes were reversible when MgADP was completely washed out, but full recovery was not achieved in these experiments because of very slow time course of ADP release (see Tanner et al., 1992). Upon

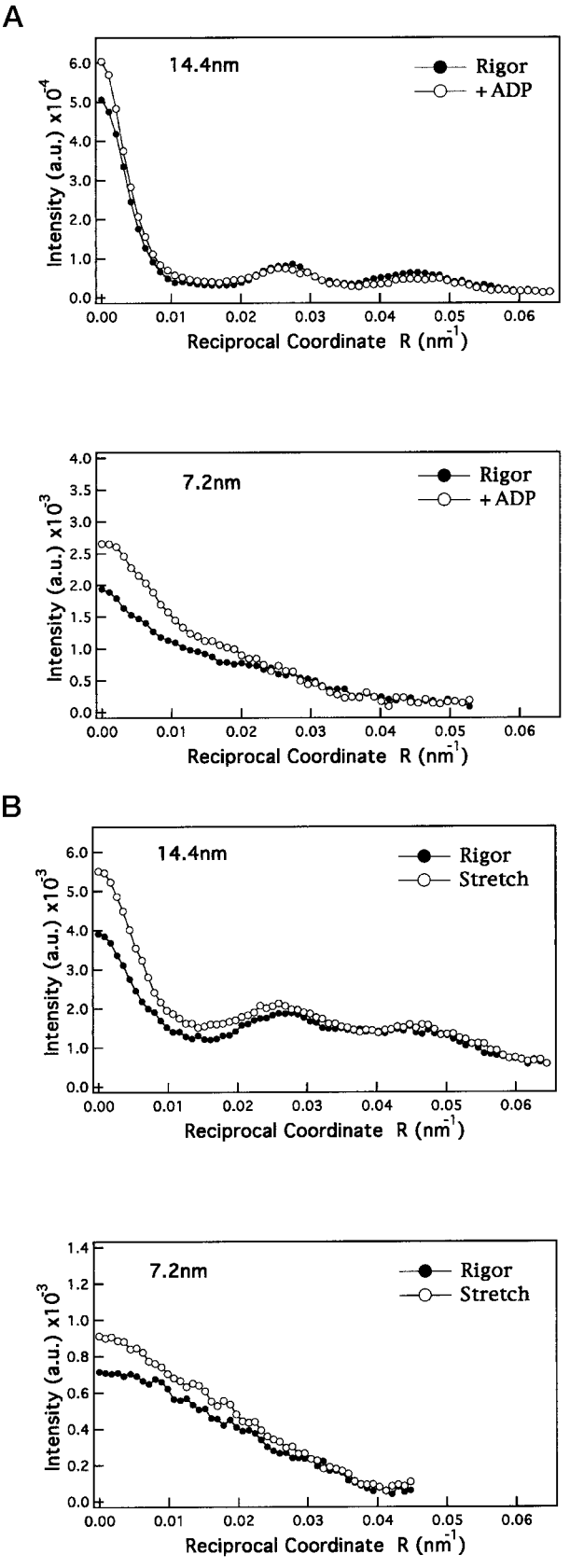

FIGURE 3 (*A*) 14.4- and 7.2-nm meridional intensity profiles along the equator from rigor rabbit muscle fibers in the absence (●) and presence of MgADP (○). (*B*) Those from rigor frog muscle before (●) and during the 4.5%-L_0 stretch (○).

applying the 4.5%-L_0 stretch (Fig. 3 *B*), the 14.4- and 7.2-nm reflections were intensified by ~33 and 17%, respectively, in the same radial range as in the ADP case. Intensity changes were too small to observe in the higher order reflections in both cases (not shown here). The reflection widths in both directions parallel and perpendicular to the meridian were mostly unchanged (see Fig. 7), implying that the ADP binding and also applied stretch did not exert any large influence on the structural order giving rise to a 14.4-nm periodicity. Lattice sampling pattern and layer-line intensities away from the meridian remained unchanged by the addition of MgADP or stretch, showing no major changes in the lattice structure. Thus, as seen in Fig. 3, the observed intensity changes were confined mostly to the meridional region. The characteristic layer lines in the rigor pattern, which are dominated by the geometry of the actin helix, are generated by the labeling of actin by all myosin heads (Cooke and Franks, 1980; Thomas and Cooke, 1980; Lovell and Harrington, 1981). Because the intensities of the 14.4-nm-based meridional reflections are still relatively high in the presence of the features of the rigor state, it is likely that there is a significant contribution to these reflections from the attached cross-bridges as affected by the original repeat of the thick filaments (see Haselgrove and Reedy, 1978; Squire and Harford, 1988). The thick filament backbone also contributes to these reflections (Huxley and Brown, 1967; Haselgrove, 1975). However, the off-meridional intensity peak on the layer line at ~1/3.9 nm^{-1} (corresponding to the eleventh order of the 43-nm basic repeat of the thick myosin filaments) (Fig. 1), which presumably comes from the backbone (Hanson et al., 1971; Bennett, 1976, cited in the book by Squire, 1981), remained unaltered in both cases (Fig. 4), suggesting that the backbone structure changed little both in the ADP binding and during stretch though it was slightly elongated by stretch (see below). The intensity increase in the 14.4-nm-based reflections upon the binding of ADP cannot be attributed to dissociation or relaxation of the cross-bridges (see below). Thus, large part of the observed intensity changes of the 14.4- and 7.2-nm meridional reflections is likely caused by an axial density alteration of the attached heads projected onto the fiber axis. The intensity increase on the meridian in these reflections would result from concentrated distribution of mass projection onto the fiber axis. Recently, Takemori et al., (1995) reported similar changes in the 14.4- and 7.2-nm reflections from a skinned frog rigor fiber upon the addition of MgADP. It is likely that a change in cross-bridge structure was induced by the external stretch, similarly to that induced by the binding of ADP, although the fractional intensity changes of the two reflections tended to differ between the two cases (see Discussion).

Fig. 5 compares intensity profiles of the 5.9- and 5.1-nm actin-based layer lines observed in the absence and presence of MgADP (Fig. 5 *A*) and before and during stretch (Fig. 5 *B*). In the rigor state, the intensities of the 5.9- and 5.1-nm layer lines are greatly enhanced by the periodical attachment of the most of cross-bridges in accordance with the

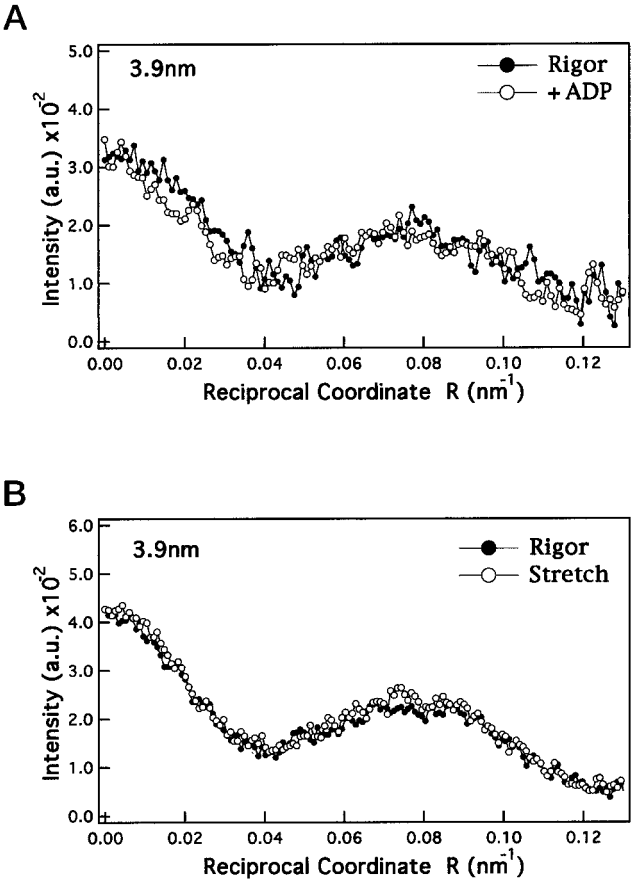

FIGURE 4 (*A*) Intensity profiles of the 3.9-nm meridional reflection (the eleventh order of the 43-nm basic repeat of the thick filament) along the equator from rabbit rigor muscle fibers in the absence (●) and presence of MgADP (○). (*B*) Those from frog rigor muscle before (●) and during the 4.5% L_0 stretch (○). This reflection is thought to come from the backbone structure of the thick filament.

actin symmetry (Huxley and Brown, 1967). Particularly, the center of gravity of the 5.9-nm layer-line distribution shifts toward the meridian by the attachment of myosin heads along the helical track with this pitch. Upon binding ADP (Fig. 5 *A*) or applying stretch (Fig. 5 *B*), there was almost no appreciable change in the 5.1-nm layer-line intensity but a small but significant decrease in the 5.9-nm layer-line intensity. The lack of distinct intensity changes of the 5.1-nm and the first actin layer lines as well as the equatorials proved no sign of detachment of the myosin heads from actin. In the 5.9-nm layer line the intensity changes were taking place in the inner region away from the peak while retaining the intensity profile of its outer part and were similar (6–8% decrease, as measured in the radial range of $0 \leq R \leq 0.05$ nm^{-1}) between in the ADP binding and during stretch, resulting in a slight outward shift of the centroid of the layer-line profile. This shift suggests an apparent decrease in the radius of the left-handed genetic helix with a 5.9-nm pitch. Application of stretch to the fibers caused a slight sharpening of the axial profile with an increased peak intensity in the higher radial part of these layer lines, whereas their integrated intensity appeared un-

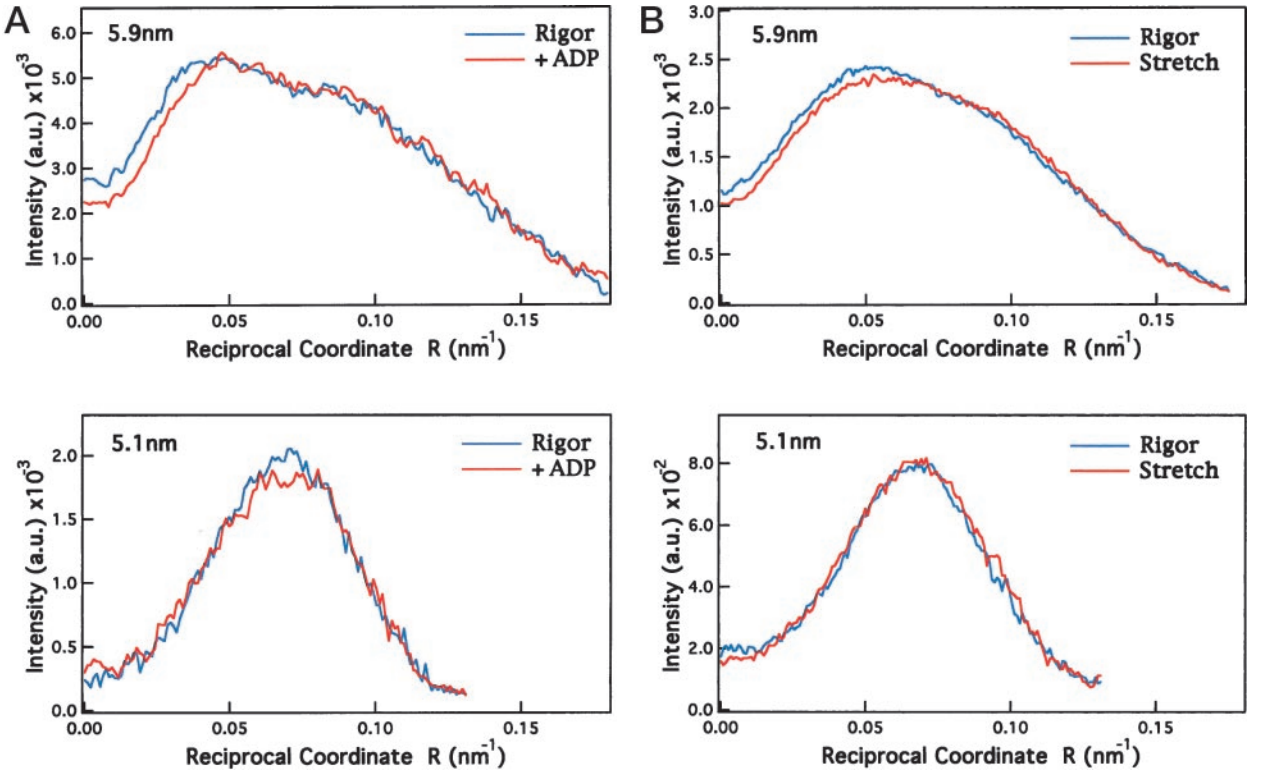

FIGURE 5 (*A*) Intensity profiles of the 5.9- and 5.1-nm actin-based layer lines along the equator from rabbit rigor muscle fibers in the absence (*blue curve*) and presence of MgADP (*red curve*). (*B*) Those from frog rigor muscle before (*blue curve*) and during the 4.5%-L_0 stretch (*red curve*). Note that a small decrease in the 5.9-nm intensity occurs in the portion from the peak to the meridian upon adding MgADP and applying the stretch, whereas no appreciable changes are observed in the 5.1-nm intensity.

changed (Fig. 1 *B*; data not shown). This effect was found after smaller stretches were applied, and thus the effect is probably caused by better alignment of the decorated actin filaments. However, little change in the axial width of the 2.7-nm reflection was detected during stretch (see Fig. 6 *B*), indicating no significant change in the helical order of the actin filaments. Although better alignment of the actin filaments affects the layer-line intensity profile, it is not responsible to the inner intensity change on the 5.9-nm layer line. This may also be supported by the fact that the 5.1-nm layer-line intensity was almost unchanged upon stretch.

Therefore, in both cases the small intensity change of the 5.9-nm layer line close to the meridian is genuine, possibly coming from the small but significant structural alteration of the attached cross-bridges in the outer portion, because the inner intensity distribution of this layer line in the rigor state is shown to be sensitive to or be much influenced by the structure of the outer portion of an attached head (Miller and Tregear, 1972; Holmes et al., 1980; Namba et al., 1980). In the case of the ADP binding, this implication is consistent with a view from the measurements of mechanical stress relaxation (Schoenberg and Eisenberg, 1987) and osmotic stress applied to a rigor muscle (Xu et al., 1993) that the ADP-induced conformational change in a myosin cross-bridge may be localized in the portion away from the actin-binding region. Thus, it is likely that the myosin heads altered their attached conformation such that the distal portion moves or flexes away from the 5.9-nm pitch-helical track, causing an apparent shift of the center of mass of the attached head closer to that of the catalytic domain, when they bind MgADP and when they are strained by stretch (see below). However, the magnitude of the conformational changes must be not as large as to bring about appreciable changes in the lateral profile of the 5.1-nm layer-line reflection and the equatorial intensities. The smallness in the observed change would be because the mass involved in the movement is a relatively small fraction of the total cross-bridge mass. It is worth mentioning that the intensity changes of the 14.4-nm-based reflections in a rigor muscle induced by stretch were in the opposite direction to those during stretch applied to an actively contracting muscle (Tanaka et al., 1991; Yagi et al., 1996), whereas the intensity change of the 5.9-nm reflection was in the same direction as it was in an actively contracting muscle (Takezawa, Sugimoto, Kobayashi, and Wakabayashi, in preparation). The relaxed muscle produces little change in any reflection during the oscillatory length changes, indicating that the rigor linkages are needed to cause the intensity changes. These results suggest strongly that the axial orientation of the distal portion of the attached heads is different between active and rigor states as argued previously for fluorescent polarization signal changes (Irving et al., 1995; Berger et al., 1996), and its displacement or flexing by stretch occurs in similar directions in both states, leading to an inner

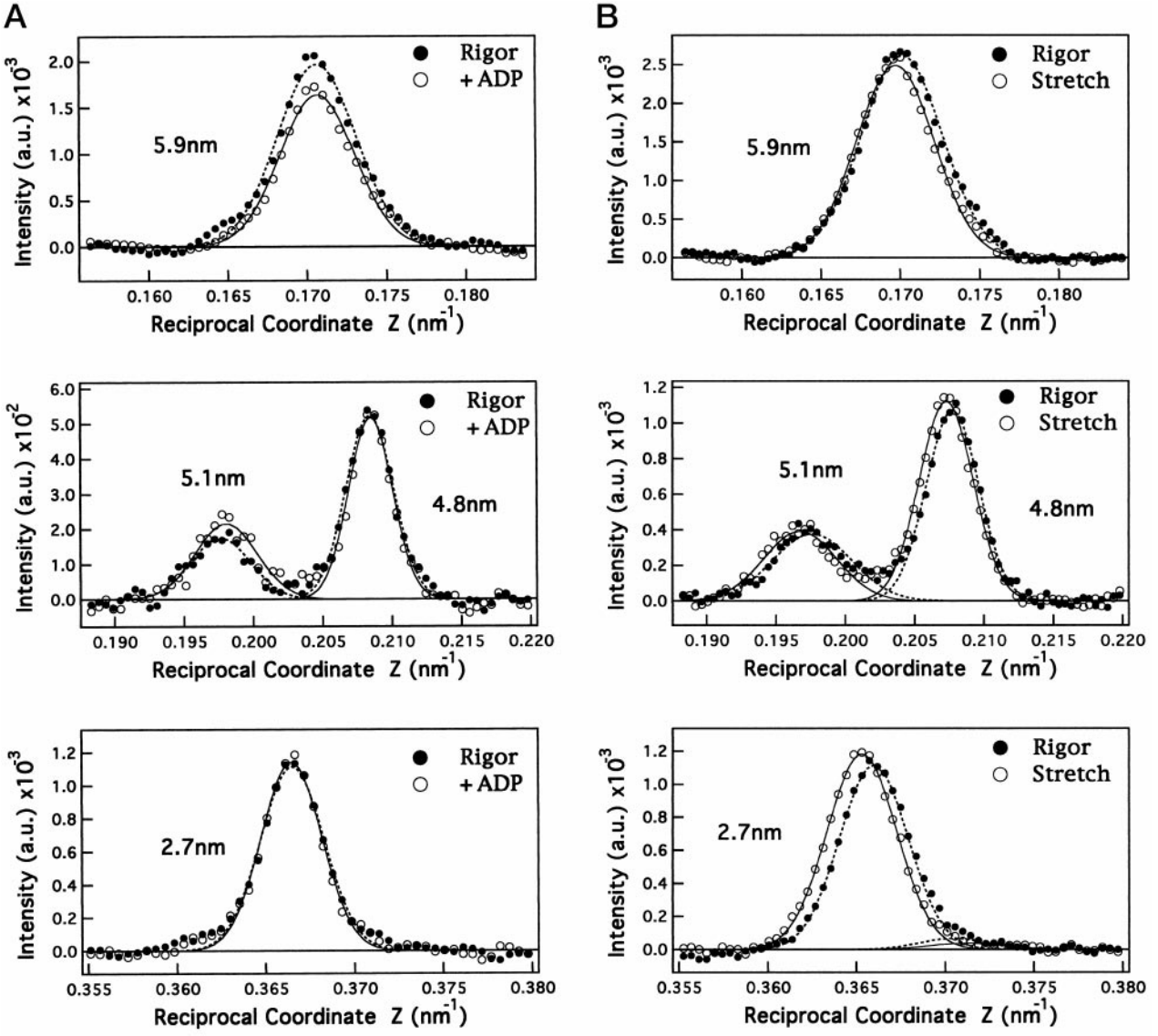

FIGURE 6 (*A*) Axial intensity profiles around the 5.9-, 5.1-nm layer lines, and the 2.7-nm meridional reflection from rabbit rigor muscle fibers in the absence (●) and presence of MgADP (○). (*B*) Those from frog rigor muscle before (●) and during the 4.5%-L_0 stretch (○). In *A* and *B*, the intensity data in the narrow range of $0 \leq R \leq 0.018$ nm^{-1} closest to the meridian were integrated (see Materials and Methods) and plotted along the fiber axis. *Z* denotes the reciprocal axial coordinate. Dashed and solid curves show the results of the Gaussian deconvolution for each reflection. Axial spacings of the actin-based reflections were derived by determining their centers of gravity of the peaks. In the 5.1-nm reflection panel, 4.8 nm is the third order reflection of the 14.4-nm repeat.

intensity decrease in the 5.9-nm layer line. Thus, the intensity increase of the 14.4-nm-based reflections observed upon stretch and binding ADP indicates that the distal portion of the attached cross-bridge that flexes away from the 5.9-nm pitch-helical track moves toward the plane perpendicular to the fiber axis, becoming more aligned with a thick filament repeat.

Axial spacing changes of the meridional and layer-line reflections induced by the binding of MgADP and by the application of stretch

The effects of MgADP binding and rapid stretches on the axial spacings of the meridional and layer-line reflections from rigor muscles were examined. Fig. 6 shows the axial intensity traces of the 5.9- and 5.1-nm layer-line and 2.7-nm meridional reflections, which were obtained by radial integration over the range of $0 \leq R \leq 0.018$ nm^{-1}, closest to the meridian (see Materials and Methods). The centroids of the reflection profiles were determined by the Gaussian deconvolution method to an accuracy better than 0.01% (Wakabayashi et al., 1994). The changes of the centroids of all but one reflections were too small to measure when MgADP was added to rigor muscle (Fig. 6 *A*). In contrast, the application of stretches shifted prominently the reflection centroids toward the low-angle side (Fig. 6 *B*).

Fig. 7 shows the intensity traces of the 14.4-nm-based meridional reflections that were obtained by radial integra-

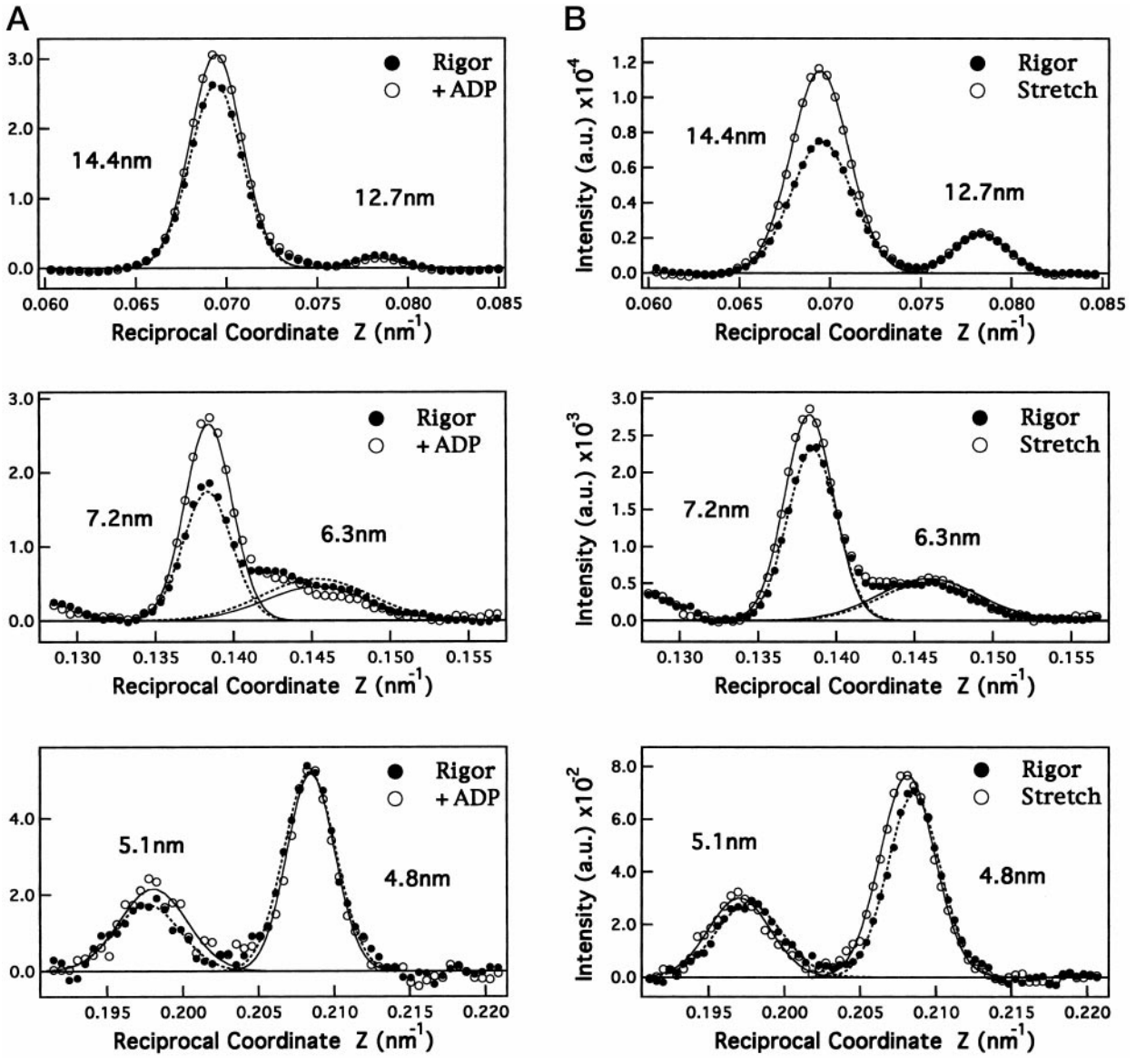

FIGURE 7 (*A*) Axial intensity profiles of the first (14.4 nm), second (7.2 nm), and third (4.8 nm) order meridional reflections of the 14.4-nm repeat from rabbit rigor muscle fibers in the absence (●) and presence of MgADP (○). (*B*) Those from frog rigor muscle before (●) and during the 4.5%-L_0 stretch (○). In *A* and *B*, the intensity data were integrated in the radial range of $0 \leq R \leq 0.022$ nm^{-1} covering the main meridional profiles. Dashed and solid curves show the results of the Gaussian deconvolution, and the axial spacing of each reflection was determined as in Fig. 6. In the 14.4- and 7.2-nm reflection panels, 12.7 nm and 6.3 nm are the third and sixth order reflections from the troponin repeat, respectively.

tion over the range of $0 \leq R \leq 0.022$ nm^{-1} covering the main meridional profiles. Upon the addition of MgADP (Fig. 7 *A*), the spacings of these reflections decreased by a small but measurable extent. Distinct shifts toward the low-angle side were observed in these reflections upon the application of stretch (Fig. 7 *B*), showing an increase in the axial spacing in real space. In Fig. 8, the spacing changes of all these reflections are summarized as bar graphs. As shown in Fig. 8 *A*, the spacings of the actin-based reflections changed little, but the 5.1-nm reflection showed a decrease of ~0.2%, and the 7.2- and 4.8-nm myosin-based reflections showed a decrease in spacing by ~0.08 and 0.03%, respectively, upon the binding of MgADP, although that of the 14.4-nm reflection changed little. In contrast, when the 4.5%-L_o stretch was applied, the spacings of the 2.7-nm reflection and 5.1- and 5.9-nm layer lines clearly increased on average by 0.17, 0.27, and 0.13%, respectively, and those of the 14.4-nm-based meridional reflections increased by 0.19% on average (Fig. 8 *B*). The spacing changes of the myosin-based reflections were slightly variable from reflection to reflection in which the change of the 14.4-nm reflection spacing was smallest in both cases (see below and Huxley et al., 1994). The spacing of the 3.9-nm meridional reflection, which was referred above, showed a parallel change with those of the 14.4-nm-based reflections; the same shift was also observed in the off-meridional

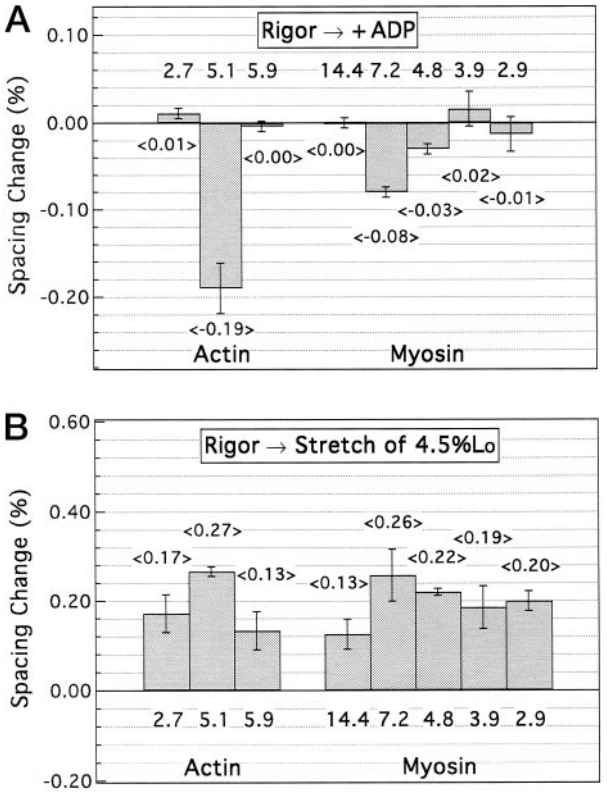

FIGURE 8 (*A*) Axial spacing changes of the first (14.4 nm), second (7.2 nm), and third (4.8 nm) order meridional reflections of the 14.4-nm repeat upon the addition of MgADP to rabbit rigor fibers. (*B*) Those of the 2.7-nm meridional reflection, 5.1-, and 5.9-nm layer lines together with the first, second, third, and fifth (2.9 nm) order reflections of the 14.4-nm repeat and the 3.9-nm reflection by the application of the 4.5%-L_0 stretch to frog rigor muscle. In *A* and *B*, the changes are expressed relative to the spacings in the rigor state, respectively. Each set of the bar graph is the mean of three observations, each of which came from one muscle. The bar shows the associated SEM. The average values are given in brackets.

subsidiary maximum. According to our recent results (Takezawa et al., 1998; Takezawa et al., in preparation), the average values of all these reflection spacings changed linearly with the magnitude of the applied stretches (i.e., tension increments), indicating that the underlying elasticity of the thin and thick filaments in the rigor lattice is Hookenian. When the tension at a 4.5%-L_o stretch was scaled to a 100% active tension (see Materials and Methods), the spacing increases of the 2.7-nm actin-based and the 14.4-nm-based meridional reflections amounted to ~0.32 and 0.40%, respectively, corresponding to 3-nm extension of the thin and thick filaments per half sarcomere. These results show that the compliance of both thin and thick filaments in a rigor sarcomere is nearly identical to that estimated in an active muscle (Wakabayashi et al., 1994; Huxley et al., 1994; Higuchi et al., 1995). Although it is not straightforward to estimate the cross-bridge compliance itself (see Discussion), the attached heads can also deform elastically in response to the stress or the binding of ADP (Tawada and Kimura, 1986; Irving et al., 1992; Suda et al., 1995; Lombardi et al., 1995). The filament extension may have a relation with different fractional intensity changes of the 14.4- and 7.2-nm reflections from those upon the binding of ADP. There also seems to be a difference in the fractional spacing change of the different myosin-based reflections. This may be attributed to an alteration of the sampling function caused by a change in the distance between the two cross-bridge regions in the A-band as well as to a different contribution of the cross-bridge and backbone form factors.

It is noteworthy that there was a differential spacing change in the three actin-based reflections when applied the 4.5%-L_o stretch (Fig. 8 *B*). This finding reveals that passive elongation of the thin filaments in the rigor muscle associated with a twisting change in the actin helical structure, as argued in a contracting muscle (Wakabayashi et al., 1994). For instance, the right-handed genetic helix is unwound, leading to a filament rotation of ~60° at its pointed end when the tension was scaled to a 100% active value. The extension and twisting natures of the actin filaments in a rigor muscle are very similar to those of the filaments observed in an actively contracting muscle (Wakabayashi et al., 1994; Huxley et al., 1994), implying that the mechanical properties of the actin filaments do not depend upon the states of muscle.

DISCUSSION

Active force of muscle is thought to be generated by a change in the configuration or a movement of cross-bridges, coupled to ATP hydrolysis cycle. Efforts have been made to identify the intermediate states in this cycle and to determine their structures. The present x-ray diffraction provides strong evidence that the chemical and mechanical states produced by an addition of MgADP (a product of ATP hydrolysis) and by the application of stretch to rigor muscles have a conformation similar to each other but different from that in the rigor state.

Possible structural changes of the attached cross-bridges

Mechanical measurements (Rodger and Tregear, 1974; Marston et al., 1976; Schoenberg and Eisenberg, 1987; Dantzig et al., 1991; Xu et al., 1993; Allen et al., 1996) have shown that the binding of ADP to the attached myosin heads results in a small reduction (10–20%) in rigor tension and radial rigor stiffness, implying that some structural alteration rather than detachment is induced in attached cross-bridges. An early x-ray diffraction study failed to detect any corresponding change, probably because of the smallness of the change and longer times over which the x-ray experiments were run (Rodger and Tregear, 1974). However, small but significant intensity changes of the 14.4-nm-based meridional reflections and of the 5.9-nm actin-based layer line reflecting underlying structural changes were clearly shown by our present x-ray quantitation. Upon the binding

of ADP, similar changes in the 14.4-nm-based reflections have already been observed by Takemori et al. (1995) for a frog skinned fiber, and most recently concerted results were also reported by Xu et al. (1998) for skinned rabbit psoas fibers at 4°C using synchrotron radiation.

In the present study, it was shown that the application of stretch to rigor frog muscle produced intensity changes similar to those brought about by the binding of ADP in the x-ray pattern of rigor rabbit muscle. The effects by stretch and ADP binding to rigor fibers are the coordinated changes in the 5.9-nm actin-based reflections and the 14.4-nm myosin-based reflections, suggesting that these two effects on the x-ray intensities can be related to each other. However, the intensity changes of the 5.9-nm layer line that are significant are quite small (at most ~8% decrease) and confined to the inner part close to the meridian. Although the 14.4- and 7.2-nm reflections change considerably, the major intensity changes of the meridional reflections with a 14.4-nm-repeat are restricted mostly to these innermost two reflections. Thus, a small localized or restricted change in the structure of an attached cross-bridge may suffice to cause the intensity changes. In the rigor state, it is thought that relatively strong intensities of the 14.4-nm-based meridional reflections relate to docking of the distal part of a cross-bridge with the thick filament repeat. Although this docking is relatively weak in rigor, when the stretch is applied or when ADP binds, more cross-bridge mass in the distal portion would become aligned with the thick filament origins, contributing to an intensity increase in the 14.4-nm-based reflections. As mentioned previously, the intensity response of the 14.4-nm reflection to a sinusoidal length changes is reverse between rigor and contracting muscles. This intensity reversal is quite consistent with that of polarization signals from fluorescence probes bound on the regulatory light chain of the myosin head in a skeletal muscle (Irving et al., 1995) reporting that stretching the fibers in rigor changes the orientation of the light chain region of the attached head in the direction away from the fiber axis. In contrast, rhodamine probes on the Cys707 residue in the catalytic domain of the head do not change their orientation in rigor during similar length changes (Irving et al., 1995). The coordinated intensity decrease in the 5.9-nm layer line close to the meridian can be made out by such a movement of the distal portion of the cross-bridge, because the intensity of this portion in the rigor state has shown to be much influenced by the structure of the outer portion of an attached cross-bridge as mentioned above. This rearrangement implicating a backward rotation or a lever-arm-like movement would make the mass distribution of the heads projected onto the filament axis narrow, but do not affect the mass distribution projected onto the plane perpendicular to the fiber axis, because the equatorial intensities remained mostly unchanged. If it is the case, our x-ray results upon stretch are consistent with the early x-ray result by Naylor and Podolsky (1981).

Preliminary modeling structural changes of the attached cross-bridges

From several lines of evidence, the observed changes are in the correct direction to the explanation that the stretch and MgADP-induced structural changes or rearrangements are related to some backward movements of the distal portion of the attached cross-bridge and do not affect the binding portion of the head to actin. The detailed modeling studies would be needed to substantiate it. However, it is not straightforward to quantify the magnitude of the conformational change from such small and restricted intensity changes. Furthermore, the rigor structure of the thin filaments in a vertebrate muscle is not fully understood: an understanding of how the cross-bridges maintain a 14.4-nm repeat and attach to actin at an incommensulate repeat of ~37 nm. In vertebrate muscle the binding pattern of myosin cross-bridges on the actin filaments is not so regular as in invertebrate muscle (Holmes et al., 1980; Namba et al., 1980) and is of some statistical nature (Haselgrove and Reedy, 1978; Squire and Harford, 1988). Thus, the function describing the actin occupancy by myosin has not been determined. Furthermore, the two attached heads of each myosin cross-bridge are strained in rigor muscle (e.g., Arata, 1990). In addition, the 14.4-nm-based meridional intensities are determined by interference between the cross-bridges and the backbone structure of the thick filaments. We have no information as regards the phase relation between them and also little information about the bound structure of the regulatory proteins, particularly troponin. These make the precise modeling difficult to simulate. At the present stage, instead of performing a quantitative modeling, we tried to see how the observed intensity changes can be shown up by using an unstrained docking structure of skeletal myosin S1 on the actin filament, which was derived by Mendelson and Morris (1997). Starting from the full-decorated S1 structure without regulatory proteins, and assuming that the actin occupancy by S1 is equal to or less than 0.6, the full decorated structure was modulated by a probability function for the binding with a 14.4-nm myosin repeat or a multiple of it (e.g., Yagi, 1996). Under such a statistical decoration over the actin filaments, modeling of the conformational changes of the attached S1 has been conducted to simulate the observed intensity changes. The amino acids of the actin and S1 molecules were built up by spherical balls with a 0.6-nm diameter. The possible structure induced by stretch or upon the binding of ADP was examined by changing the orientation of the distal portion of bound heads. In the calculation, the partially decorated actin filament structure was directly transformed without the use of the helical symmetry. To reduce the strong interference between the actin and attached S1, which appears on the 5.1-nm and 5.9-nm actin layer lines, it was necessary to fluctuate the distal portion of the attached heads around the fixed angle. Our present modeling suggested that the observed intensity change of the 5.9-nm layer line could be realized by an axial (backward) rotation

of an attached S1 tail portion by 10–20° with 20–30° disorder toward the M-line of the sarcomere, corresponding to a 1–2 nm movement of the tip of an α-helical tail. Thus, such a backward rotation or movement leads to the intensity changes of the 5.9-nm actin reflection and the 14.4-nm-based meridional reflections as in the observed manner: the models revealed that the center of mass of the bound heads shifted closer to that of the catalytic domain, and more mass in the light chain-binding domain became aligned with a thick filament-repeat. Fig. 9 depicts an example of our modeling conformational changes of attached heads (Fig. 9 *A*), and calculated intensity behaviors of the 5.9- and 5.1-nm layer lines and the 14.4-nm meridional reflection (Fig. 9 *B*). The resulting large disorder reflects the wide orientation distribution of bound myosin heads on the actin filament. More detailed modeling will be reported elsewhere.

Alternative explanations

Although the 14.4- and 7.2-nm reflections change considerably in both cases, the major intensity changes of the meridional reflections with a 14.4-nm repeat are restricted to these inner two reflections. Also, the intensity change of the 5.9-nm layer line is very small and confined to the part close to the meridian. No significant intensity changes were found in the other actin-based reflections as well as the myosin-based reflections. It may be unlikely that a structural change in a cross-bridge produces intensity changes of only a small number of reflections. Thus, the observed x-ray changes may be caused by a structural alteration other than that described above.

As mentioned above, although a large-scale redistribution of the attached cross-bridges is unlikely, the x-ray diffraction results do not completely exclude the possibility of dissociation or relaxation of cross-bridges upon the binding of ADP or by stretch, because an ADP binding or stretch may reduce the affinity of cross-bridges for actin, slight relaxation leading to affect the intensity of the 5.9-nm layer line, particularly in the inner region near the meridian (e.g., Kim et al., 1998). The intensity decrease of the 5.9-nm layer line is at most ~8% in both cases. This value may represent, if any, ~4% detachment of cross-bridges, which could be escaped to detect in EPR and birefringence measurements. However, such a small detachment can not explain a large intensity increase in the 14.4- and 7.2-nm reflections. Furthermore, Xu et al. (1998) reported that upon the binding of ADP the intensity increase of the 14.4- and 7.2-nm reflections occurred even at low temperature where relaxation would lead to a decrease in these reflection intensities. During stretch the actin filaments are elongated and this elongation accompanies by a slight twisting of the left-handed helices. The apparent outward shift of the 5.9-nm layer-line centroid (Fig. 5 *B*) can be explained if such a twisting causes a decrease in a radius of the left-handed genetic helix. However, the same change of this layer line is seen upon the binding of ADP (Fig. 5 *A*) where the tension decreases. As evidenced from a slight decrease in the axial reflection width and an increase in the axial reflection spacing upon stretch, the increased cross-bridge order and an alteration of the thick filament backbone structure including an S2 part may modify the meridional intensities of a 14.4-nm repeat without changing the cross-bridge structure, because interference between the cross-bridges and the backbone structure governs their intensities. It is uncertain whether such better regularity of the backbone structure and cross-bridges can make out solely the intensity changes of these first and second order reflections with a 14.4-nm repeat as observed. Alternatively, the shift in the axial spacing of the thick filaments may be caused by a change in the distance between the two cross-bridge regions and alters the sampling function along the meridian in the x-ray pattern, leading to the intensity changes.

Relationship to other work

The recent three-dimensional reconstructions of cryoelectron microscopy images show a large conformational change or backward movement in the distal portion of a smooth muscle S1 decorated in the actin filaments in the presence of MgADP (Whittaker et al., 1995). But this same change has not been seen using a skeletal muscle S1 (Jontes and Milligan, 1997). Our modeling suggests that the magnitude of the changes expected on the basis of our x-ray results is not as large as those shown up in such a reconstructed image, consistent with the recent x-ray results on the thin actin filaments in an overstretched muscle in which the actin filaments were fully decorated exogenously with skeletal S1 by Poole et al. (1998). A number of spectroscopic probe studies (Arata, 1990; Hambly et al., 1991; Allen et al., 1996; Gollub et al., 1996; Baker et al., 1998) have suggested that unlike in a smooth muscle S1, little or very small movement occurs in the light chain-binding domain of a skeletal muscle S1 in decorated muscle fibers or intrinsic myosin heads in striated muscle fibers upon the binding of ADP. Possible explanations for this difference between smooth and skeletal S1s have been discussed (Gollub et al., 1996; Barsotti et al., 1996). In contrast, a rotary-shadowing EM study on the skeletal myosin molecules in solution suggested that there exist the heads with a straight form in equilibrium with those with a bent form and the population of heads with a bent form increases in the presence of ADP (Tokunaga et al., 1991). In addition, recent x-ray solution scattering studies have revealed a significant decrease in the radius of gyration (R_g) of a chicken skeletal muscle S1 in the presence of MgADP. Although the change in R_g (~0.2 nm) was less than that (~0.3 nm) observed in the presence of ATP, the analysis shows that sizable bending of the molecule is occurring upon the binding of ADP (Sugimoto et al., 1995, 1996; Sugimoto, Tokunaga, and Wakabayashi, submitted for publication). Most recently, Katayama (1998) using a quick freeze deep-etch EM technique showed that in the presence of ADP the myosin heads

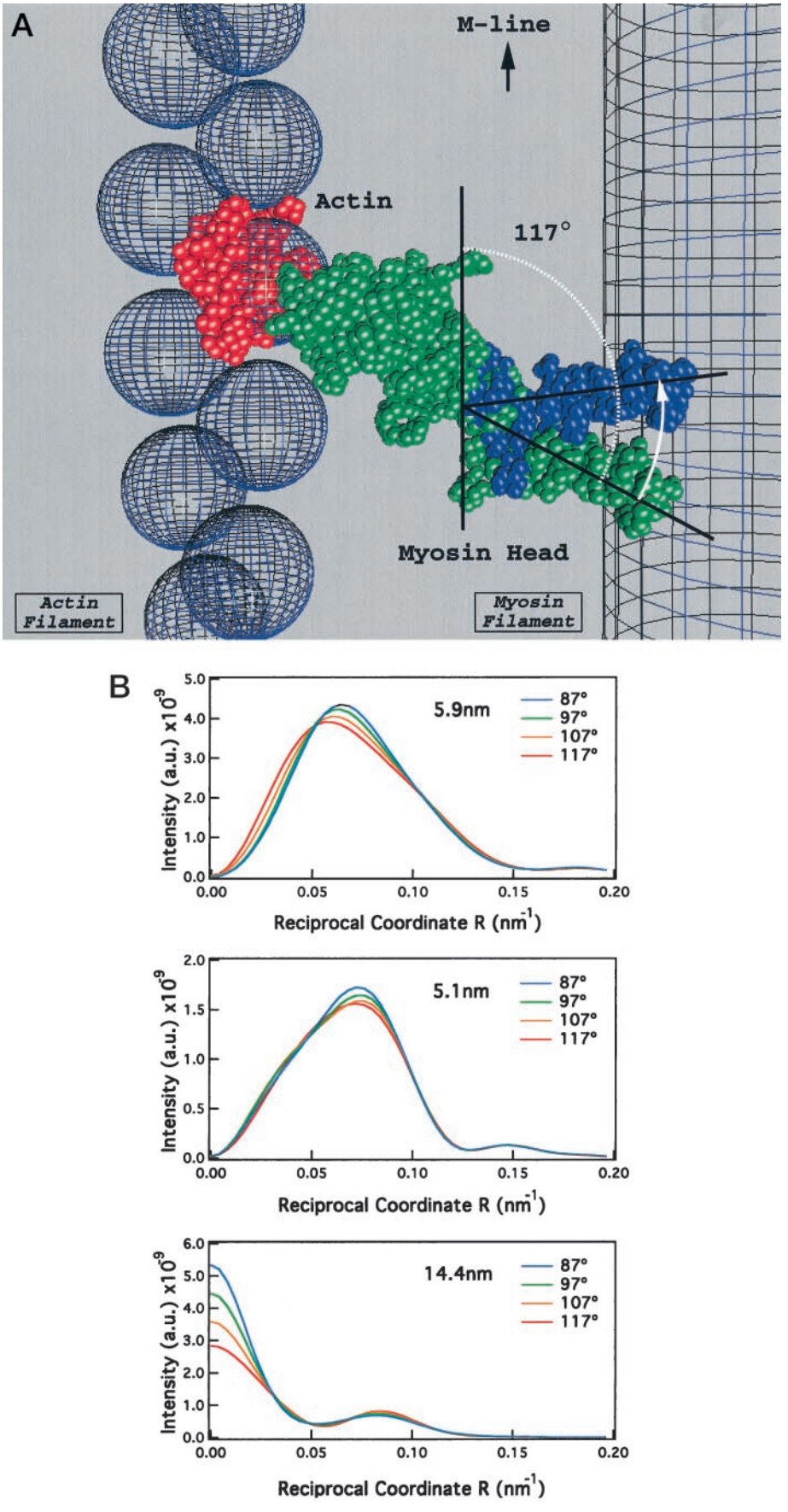

FIGURE 9 An example of modeling structural changes of the myosin head bound to actin, which explains the observed intensity changes. (*A*) Modeling using the atomic structures. The F-actin structure decorated by S1 (Mendelson and Morris, 1997) was taken from the Protein Data Bank (identification code; 1alm.pdb), in which one actin (*red*)-S1 (*green*) complex is shown by the atomic structures. In our present modeling (see text), the light chain-binding region was rotated at around a Cys707 residue toward the M-line of the sarcomere while retaining the orientation of the catalytic domain in order to produce the observed intensity profile change in the 5.9-nm layer line (blue color). In the initial structure, the angle (θ) of the long axis of the light chain-binding region against the filament axis is ~117° with about 30° disorder. (*B*) Calculated intensity behavior of the 5.9- and 5.1-nm layer lines and the 14.4-nm meridional reflection when the angle, θ, was varied from 117° toward the M-line. The angle changes of 10–20° may match the observed intensity changes.

of skeletal heavy meromyosins attached to the actin filaments appeared rounded and that a large fraction of heads were tilted to the anti-rigor orientation, very consistent with the present x-ray interpretation.

Earlier structural studies have suggested that the application of stretch or external loads does not alter the overall structure of rigor cross-bridges and some compliant elements are only extended (dos Remedios et al., 1972; Naylor and Podolsky, 1981; Cooke, 1981; Podolsky et al., 1982; Hambly et al., 1991; Burghardt et al., 1997). The present x-ray measurements, however, indicated most probably that the application of stretch to rigor muscle caused a conformational change or a backward movement in the light chain-binding domain similar to that induced upon the addition of MgADP. Such conformational changes may be also consistent with the recent results of Irving et al. (1995) and Sabino-David et al. (1998) who observed a small but significant change in the orientation of fluorescent probes attached to the myosin light chain when the rigor muscle fiber was stretched. The changes also include the local change around a Cys707 residue on the catalytic domain of a head (Fajer et al., 1990; Burghardt et al., 1997) as well as in an S2 part. Such movements may also explain the oscillatory intensity changes of the 14.4-nm reflection when slow (Tanaka et al., 1991) and fast (Yagi et al., 1996) sinusoidal length perturbations were applied to rigor muscles. The smallness in the observed change would be because of the mass involved in the movement is only a small fraction of the total cross-bridge mass. These relations indicate that the myosin cross-bridge can elastically deform.

CONCLUSION

The present x-ray measurements showed that small but significant intensity changes of the actin and myosin-based reflections were induced when stretch was applied and MgADP bound to rigor muscles. These coordinated changes indicate that stretch and ADP binding seem to mediate similar structural changes (i.e., a backward rotation or a lever-arm-like movement) in a light chain-binding domain of the attached myosin head. The observations may provide a further insight into energy transduction in muscle; both chemical (ADP binding) and mechanical (stretch) interventions can reverse the contractile cycle by causing a backward movement of this domain of attached heads in the rigor state.

What is distressing about the x-ray observations is their apparent discrepancy to the EPR and fluorescent polarization studies that detected little and very small movement of probes in skeletal muscle. Although the spectroscopic data do not provide exclusive explanation (see Sugi, 1998), this discrepancy remains to be resolved by additional studies.

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