The Effect of Thin Filament Activation on the Attachment of Weak Binding Cross-Bridges: A Two-Dimensional X-Ray Diffraction Study on Single Muscle Fibers

T. Kraft,* S. Xu,# B. Brenner,* and L. C. Yu#

*Molekular- und Zellphysiologie, Medizinische Hochschule Hannover, D-30625 Hannover, Germany, and #National Institutes of Health, Bethesda, Maryland 20892 USA

ABSTRACT To study possible structural changes in weak cross-bridge attachment to actin upon activation of the thin filament, two-dimensional (2D) x-ray diffraction patterns of skinned fibers from rabbit psoas muscle were recorded at low and high calcium concentration in the presence of saturating concentrations of MgATPγS, a nucleotide analog for weak binding states. We also studied 2D x-ray diffraction patterns recorded under relaxing conditions at an ionic strength above and below 50 mM, because it had been proposed from solution studies that reducing ionic strength below 50 mM also induces activation of the thin filament. For this project a novel preparation had to be established that allows recording of 2D x-ray diffraction patterns from single muscle fibers instead of natural fiber bundles. This was required to minimize substrate depletion or product accumulation within the fibers. When the calcium concentration was raised, the diffraction patterns recorded with MgATPγS revealed small changes in meridional reflections and layer line intensities that could be attributed in part to the effects of calcium binding to the thin filament (increase in I_{380}, decrease in first actin layer line intensity, increase in I_{59}) and in part to small structural changes of weakly attached cross-bridges (e.g., increase in I_{143} and I_{72}). Calcium-induced small-scale structural rearrangements of cross-bridges weakly attached to actin in the presence of MgATPγS are consistent with our previous observation of reduced rate constants for attachment and detachment of cross-bridges with MgATPγS at high calcium. Yet, no evidence was found that weakly attached cross-bridges change their mode of attachment toward a stereospecific conformation when the actin filament is activated by adding calcium. Similarly, reducing ionic strength to less than 50 mM does not induce a transition from nonstereospecific to stereospecific attachment.

INTRODUCTION

In the past, many attempts have been made to characterize the structural features of the different cross-bridge states that are involved in active cross-bridge turnover in skeletal muscle. From biochemical and mechanical studies it was derived that cross-bridges can alternate between two groups of states: weak binding non-force-generating states and the strong binding force-generating states (Lymn and Taylor, 1971; Stein et al., 1979). Weak binding cross-bridge states can bind to actin only with low affinity (Stein et al., 1979; Chalovich et al., 1981), even when the thin filament is activated (Chalovich et al., 1981; Wagner and Giniger, 1981), and are therefore unable to activate the contractile system (Chalovich et al., 1983). In contrast, the actin affinity of strong binding cross-bridge states was found to be highly dependent on activation of the thin filament (Greene and Eisenberg, 1980b). Because of their much higher actin affinity when the thin filament is activated (Marston and Weber, 1975; White and Taylor, 1976; Highsmith, 1977; Margossian and Lowey, 1978; Greene and Eisenberg, 1980a; Greene et al., 1983), strongly binding cross-bridges are able to activate the contractile system, even in the absence of calcium (Bremel and Weber, 1972; Greene and Eisenberg, 1980b).

More recent data from electron microscopy (Lehman et al., 1995) showed that on the actin filament the sites for strong cross-bridge attachment are covered by tropomyosin at low calcium concentrations. Raising calcium or attachment of nucleotide-free cross-bridges induced movement of tropomyosin, uncovering sites on the actin filament for strong cross-bridge attachment. These observations provide a structural basis for the calcium dependence of the strong cross-bridge binding to actin. Furthermore, the work of Lehman and co-workers showed that tropomyosin does not interfere with the sites on actin proposed to be involved in the interaction with weakly binding cross-bridges. This is consistent with the finding that interaction of weakly binding cross-bridges with actin is affected only slightly by calcium (Chalovich et al., 1981; Wagner and Giniger, 1981).

Based on the concept that the cross-bridges alternate between two distinctly different groups of states, it was proposed that force generation and muscle shortening result from a structural change associated with the transition of the cross-bridges from a weakly to a strongly bound state. This concept, however, was recently extended on the basis of mechanical experiments: it was proposed that, in fact, two distinctly different force-generating steps can be distinguished (Brenner, 1991; Brenner et al., 1995): 1) Isometric force is generated as cross-bridges undergo a structural change at the transition from a weakly bound to a strongly

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Address reprint requests to Dr. Theresia Kraft, Molekular- und Zellphysiologie, Medizinische Hochschule Hannover, D-30625 Hannover, Germany. Tel.: 49-511-532-2734; Fax: 49-511-532-4296; E-mail: kraft.theresia@mh-hannover.de.

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bound state. 2) Quick tension recovery after a quick length change during isometric contraction seems to be due to a redistribution between different force-generating states. In both concepts, weakly attached cross-bridges are assumed to be essential intermediates for force generation. This view is supported by our previous results (Brenner, 1991; Kraft et al., 1995) showing that during active cross-bridge cycling, the weak attachment of the cross-bridges to actin is an essential prerequisite for their transition to the force-generating states.

Yet, because in this concept the transition from weak to strong cross-bridge binding is thought to result in cross-bridge strain and thus in generation of isometric force, a central question arises: What is the structure and attachment mode of weakly attached pre-force-generating cross-bridges versus force-generating cross-bridges? In other words, one relevant structure to be studied for the understanding of the force-generating mechanism is the starting structure, i.e., the complex between a weakly binding cross-bridge and the activated actin filament.

All previous two-dimensional (2D) x-ray diffraction studies of weakly bound cross-bridges were performed under relaxing conditions, i.e., when the thin filament was not activated. Under these conditions, both frog and mammalian skeletal muscle seemed to reveal no sharply defined orientation of weakly attached cross-bridges with respect to the actin helix (Xu et al., 1987, 1997). This was also supported in saturation transfer EPR experiments, which showed an increase in rapid motions of an EPR probe on weakly attached myosin heads (Fajer et al., 1991; Berger et al., 1989). Such a disordered appearance of weakly attached cross-bridges in the absence of calcium was also found for cross-bridges weakly attached to unregulated actin by electron microscopy of negatively stained or frozen hydrated samples of the actomyosin complex in solution. In most of these studies, either with S1 cross-linked to actin (Craig et al., 1985; Applegate and Flicker, 1987) or under low ionic strength conditions (Walker et al., 1994, 1995), cross-bridges that are weakly attached to the unregulated actin were found to exist in a variety of different configurations. No preference for a specific attachment angle was observed. However, Pollard and co-workers (1993) found, using freeze-fracture and deep etching methods, that cross-bridges weakly attached to unregulated actin are indistinguishable from nucleotide-free rigor complexes. Therefore, the question remained: Is there a change in the mode of weak cross-bridge attachment to native thin filaments when the thin filaments are activated by calcium, i.e., do the weakly attached, pre-force-generating cross-bridges adopt a more ordered, stereospecific structure when the thin filament is activated?

One possibility for the study of a complex between weak binding states and the activated actin filament in muscle fibers is to use the very slowly hydrolyzable nucleotide analog MgATPγS. Previously we demonstrated (Kraft et al., 1992) that cross-bridges with MgATPγS in the presence of calcium represent weak binding cross-bridge states. Because MgATPγS is hydrolyzed only slowly, the cross-bridge cycle is essentially blocked at the hydrolysis step such that the cross-bridges cannot make a transition to the strong binding (force-generating) states. Therefore, cross-bridges with MgATPγS represent a population of weak binding states, and no detectible active force is generated in the presence of MgATPγS. Yet, most interestingly, the mechanical experiments provided evidence for a different mode of interaction between weakly binding myosin heads and activated actin versus inactivated actin. It was shown that, contrary to previous reports (Dantzig et al., 1988), calcium did not alter the actin affinity of the weakly binding cross-bridges with MgATPγS to a great extent, but that the attachment and detachment kinetics of these cross-bridges changed significantly upon the addition of calcium. The rate constant for cross-bridge detachment decreased ~100-fold, whereas the rate constant for attachment decreased some 40-fold, suggesting some difference in the mode of attachment. In the present study we therefore used MgATPγS to address the question of whether structural changes can be detected when cross-bridges in weak binding states bind to the activated instead of the inactivated thin filament.

Another possibility for the study of the complex between weakly bound cross-bridges and the activated thin filament was thought to be to lower the ionic strength to less than 50 mM in the absence of calcium (Head et al., 1995). It was proposed that at such low ionic strength the actin filament makes a transition from its blocked into the closed conformation (just as upon adding calcium), thus allowing the weak binding cross-bridges to attach to actin in the same way as when calcium is raised (Geeves and Conibear, 1995). In the present study we therefore attempted to induce such a transition of the thin filament by reducing the ionic strength from 80 mM to 30 mM under relaxing conditions (without calcium).

We used 2D x-ray diffraction of single skeletal muscle fibers to obtain structural information of cross-bridges weakly attached to the different states of the thin filament within the constraints of the three-dimensional lattice. Yet, correlation of the structural data with biochemical and/or mechanical properties is required to fully characterize the different states. Thus we had to 1) develop an approach to record 2D x-ray diffraction patterns from single fibers like those used for mechanical studies and 2) choose the same conditions for the x-ray studies that had been applied in our mechanical studies. In the past, to obtain sufficient intensity not only for recording of the equatorial reflections but also for meridional reflections and for actin- and myosin-based layer lines, fiber bundles had been used instead of single fibers. The disadvantage of using small bundles for studying various conditions is the limited diffusion within such bundles, which might lead to substrate depletion and accumulation of reaction products. In a preliminary study we had found that this can become a serious problem, even for slowly hydrolyzable nucleotide analogs such as MgATPγS. Therefore we developed a new approach that allows the use of isolated single fibers instead. To overcome the low intensities in diffraction patterns from single fibers, some 30 isolated single fibers were mounted side by side in the setup.

This "single fiber array" allows us to maintain diffusion properties of single fibers, making it possible to record 2D x-ray diffraction patterns under conditions identical to those used in mechanical or biochemical experiments.

Using this single-fiber array, we studied 2D x-ray diffraction patterns 1) in the presence of MgATPγS at low and high calcium concentrations and 2) at 80 mM and 30 mM ionic strength in the presence of MgATP without calcium. Raising the calcium concentration in the presence of MgATPγS, we found some evidence for small-scale structural changes of weakly attached cross-bridges. However, there was no indication of a transition of the weakly binding cross-bridges to stereospecific attachment to actin. Furthermore, no such transition was induced by lowering the ionic strength to 30 mM in the presence of MgATP. A preliminary account of this work was presented earlier (Kraft et al., 1995c).

MATERIALS AND METHODS

Fiber preparation

Small bundles from rabbit psoas muscle were excised and chemically skinned according to a method described earlier (Brenner, 1983; Yu and Brenner, 1989; Kraft et al., 1995a). Within a few hours after the skinning procedure, single fibers were isolated and kept in skinning solution until they were mounted for the experiment (see below). We found that keeping the muscle as single fibers instead of bundles resulted in a better preservation of the regulatory properties and a better structural stability of the fibers and allowed us to study weakly bound cross-bridges at very low ionic strength (30 mM), at low (1°C) and high (20°C) temperatures. In these preparations we found no evidence for any active cross-bridge turnover (no active force redevelopment was observed in quick-release experiments; cf. Brenner, 1983) under relaxing conditions, including 30 mM ionic strength and high temperature (20°C).

Solutions

All solutions were adjusted to pH 7.0 at the respective experimental temperature. The chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), except where noted otherwise. The ionic strength of all solutions was adjusted by adding potassium propionate.

Skinning solution, including protease inhibitors, was prepared according to a recently described method (Kraft et al., 1995).

The relaxing solution consisted of 10 mM imidazole, 3 mM $MgCl_2$, 1 mM MgATP, 1 mM EGTA, 5 mM dithiothreitol, 10 mM glutathione, 1000 U/ml catalase.

All MgATPγS solutions were prepared with ATPγS from Boehringer Mannheim (Indianapolis, IN), which was purified by ion exchange chromatography as previously described by Kraft et al. (1992). After purification, the ATPγS contained ~3% ADP, and the ATP content was below the detection limit of the high-performance liquid chromatography. However, to ensure sufficient removal of ATP in the solutions, hexokinase and glucose were added. As a result, in our MgATPγS experiments at high calcium concentration there was no detectable activation of the fibers, which would have indicated some MgATP contamination in the solutions.

The low-calcium MgATPγS solution consisted of 10 mM imidazole, 2 mM $MgCl_2$, 3 mM EGTA, 10 mM MgATPγS, 0.2 mM Ap_5A, 0.5 U/ml hexokinase, 200 mM glucose, 5 mM dithiothreitol, 10 mM glutathione, and 1000 U/ml catalase.

For the high-calcium MgATPγS solution, the EGTA of the low-calcium MgATPγS solution was replaced with 1 mM CaEGTA.

For comparison with MgATPγS we also recorded diffraction patterns in relaxing solution with 10 mM MgATP: 10 mM imidazole, 2 mM $MgCl_2$, 3 mM EGTA, 10 mM MgATP, 200 mM glucose, 5 mM dithiothreitol, 10 mM glutathione, and 1000 U/ml catalase.

Rigor solution

To quickly remove the MgATP when transferring the fibers to rigor, we used a solution containing 10 mM imidazole, 2.5 mM EGTA, 7.5 mM EDTA, and 135 mM potassium propionate. To avoid any MgATP contamination in the subsequent Mg rigor solution, we usually kept the fibers for at least 30 min in rigor solution containing 10 mM imidazole, 2.5 mM EGTA, 2.5 mM EDTA, and 150 mM potassium propionate and changed the solution several times. Then the rigor solution was exchanged for Mg rigor solution, which contained 10 mM imidazole, 2 mM $MgCl_2$, 3 mM EGTA, 200 mM glucose, 0.5 U/ml hexokinase, 5 mM dithiothreitol, 10 mM glutathione, and 1000 U/ml catalase. For Mg rigor solution at high calcium concentration, the EGTA was replaced with 1 mM CaEGTA.

Mounting of the single fibers

To record 2D x-ray diffraction patterns from isolated single fibers with sufficient intensity, it was necessary to mount ~30 single fibers side by side in the x-ray setup (Fig. 1 *a*).

The ends of the isolated single fiber segments (length ~28 mm) were attached to stainless steel holders that were covered with 3145RTV silicone glue (Dow Corning, Midland, MI). During the mounting procedure and after all 30 fibers were mounted, additional layers of silicon glue were added, so that the ends of each fiber were surrounded by silicon glue and

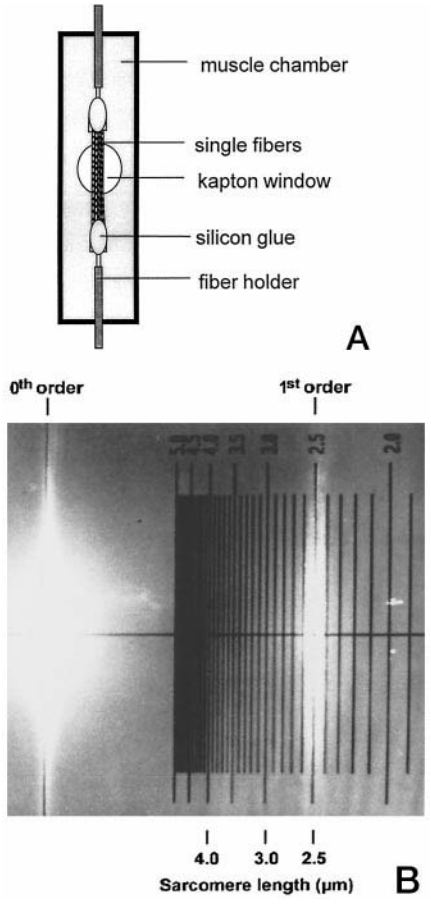

FIGURE 1 (*a*) Schematic illustration of the single fiber preparation. (*b*) Typical laser diffraction pattern of a single fiber array after adjustment of the sarcomere length to 2.5 μm.

thus firmly attached. In general, the resulting array of single fibers was ~1.2 mm wide and 0.3 mm thick and had a free length of ~22 mm. The whole procedure of isolating and mounting the fibers was carried out in skinning solution at 5°C. Special care was taken to maintain the same sarcomere length for all fibers and to mount them in parallel. For all preparations the sarcomere length was controlled by laser light diffraction and was adjusted to 2.4 μm before the experiment (Fig. 1 *b*).

The single-fiber array was mounted in a chamber that allowed us to orient the fibers vertically for the experiment. The temperature of the chamber (and thus of the solution) was controlled by two water-cooled Peltier elements. To minimize any temperature and concentration gradients, especially in the small gap between the kapton windows where the x-ray beam passed through, the solution was permanently pumped up and down (flow rate ~1 ml/min).

Beam damage dependent on exposure time

To determine the maximum acceptable x-ray exposure time for the fibers at a certain intensity of the x-ray beam, we initially performed control experiments with individual single fibers. The x-ray beam shutter was repeatedly opened for a defined time for defined irradiation of the fibers. Between openings we applied ramp-shaped stretches to the fibers under relaxing conditions and recorded (by laser diffraction) the resulting change in sarcomere length exactly at the spot irradiated by the x-rays. Fig. 2 shows the change in sarcomere length within the irradiated area as a function of exposure time times x-ray beam intensity. We found that already after the first short exposures, the sarcomere length change within the irradiated part of the fiber decreased quite dramatically under control conditions (Fig. 2). It was assumed that the decrease in length change indicates an increase in structural damage to the contractile proteins, which is presumably due to free radicals generated by the x-rays. This damage, for instance, could arise from cross-linking of myofilaments similar to what has been observed in myofibrils as a result of exciting fluorophores, which induces photooxidation (Knight and Parsons, 1991).

Therefore we tested several free radical scavengers with regard to their protective potential against the observed radiation effects. We found that the addition of 10 mM glutathione, for example, allows for much longer exposure times at a given beam intensity before we observe changes similar to those under control conditions (Fig. 2). The exposure time needed to reach 80% of the initial length change at a given beam current increases about fivefold in the presence of 10 mM glutathione. The same effect was observed with the addition of 10 mM dithiotreitol. Even better protection can be achieved by adding 30 mM glutathione or dithiotreitol (data not shown). For our experiments we finally used 10 mM glutathione, 5 mM dithiotreitol, and 1000 U/ml catalase in all experiments, for the following reasons: 1) Catalase effectively degrades H_2O_2 molecules. 2) Because glutathione contributes to the ionic strength, higher concentrations of glutathione could not be used for our experiments at low ionic strength. 3) Both dithiotreitol and glutathione tend to activate the fibers, especially under low ionic strength conditions, presumably because of impurities in the dithiotreitol and glutathione. In control experiments we found that, for example, in the presence of 10 mM dithiotreitol the force/pCa relationship of skinned psoas fibers was shifted to the left by ~0.2 pCa units. Part of the activating effect on relaxed fibers can be inhibited by increasing the EGTA concentration to 3 mM (authors' unpublished observations), suggesting some calcium contamination of dithiotreitol and glutathione.

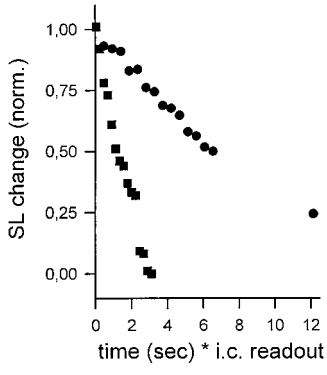

FIGURE 2 Change in responsiveness of sarcomere length (SL) to ramp-shaped changes in length of single fibers as a function of x-ray irradiation exposure time times x-ray beam intensity (= time-integrated ionization chamber readout). Relaxing conditions are recorded in the absence (■) and presence (●) of 10 mM glutathione. The length changes during ramp-shaped stretches were monitored by laser diffraction. T = 5°C. Data are from two fibers. Data are normalized to the sarcomere length change of each fiber before x-ray exposure.

The maximum acceptable exposure time was calculated by taking into account ionization chamber readout (i.c. counts) × exposure time during which the length change was reduced by 25%. This was 4 s × i.c. counts per irradiated spot. To increase maximum exposure time, the fibers were scanned up and down in the beam so that 6 mm of the muscle length were exposed to the x-ray beam (scanning speed was 2 mm/s). With a beam heigth of 0.5 mm, the usually maximum acceptable exposure time at half-maximum i.c. counts was 480 s total.

To further ensure that the results were not affected by x-ray beam damage, the diffraction patterns under different experimental conditions were recorded in random order. In general, there was no difference between diffraction patterns recorded under the same conditions at the very beginning of an experiment compared to the end of an experiment. The only exception was, if diffraction patterns were recorded under low ionic strength conditions (30 mM) at high temperature (20°C) toward the end of an experiment (after a few other exposures), occasionally the fibers were slightly activated and began to shorten during the exposure. This indicates that even very short exposure times seem to cause some damage, primarily to the regulatory proteins of the fibers, resulting in instabilities at low ionic strength.

X-ray diffraction

Preliminary studies and control experiments (MgATPγS-titration, Fig. 3 *a*) were carried out at the laboratory source (Elliott, GX-6), and the patterns were recorded on an MAR research imaging plate (X-ray Research GmbH, Hamburg, Germany). The pixel size was 50 μm × 50 μm, and the scanning area was 10 cm in diameter. The x-ray beam was focused by a double-mirror Franks camera. The specimen-to-detector distance in this setup was 89 cm, and ~80% of the x-ray path was enclosed in helium-filled tubes.

Most 2D x-ray diffraction patterns shown here were recorded using the high intensity x-ray source at the Deutsches Elektronen Synchrotron (DESY) in Hamburg, Germany. The experiments were carried out at the X-13 beamline of the European Molecular Biology Laboratory outstation. The size of the x-ray beam at the specimen was reduced to ~0.5 mm × ~1.7 mm. The specimen-to-detector distance was 2.84 m. The two-dimensional diffraction patterns were recorded on imaging plates (Fuji, Japan) and scanned at 100 μm × 100 μm pixel size with a BAS2000 scanner. At the end of each series of exposures we recorded background patterns with exactly the same configuration by removing all muscle fibers through calcium activation at high temperature until all fibers were broken. These background patterns were subtracted from the diffraction patterns to reduce the background level near the main beam, which arises from scatter of the x-rays by optical components or solution, or when passing through the kapton windows.

One set of the MgATPγS experiments was carried out at the synchrotron radiation source (SRS) in Daresbury, England, at station 2.1. The experimental conditions were the same as at the DESY, including camera configuration. The exposure times in Daresbury were shorter, because of the higher brilliance of the x-ray beam. The data were recorded with a position-sensitive 2D wire detector. Using a semitransparent beam stop in Daresbury allowed us to subtract the machine background very precisely. This then allowed us to normalize the overall intensity in these patterns to

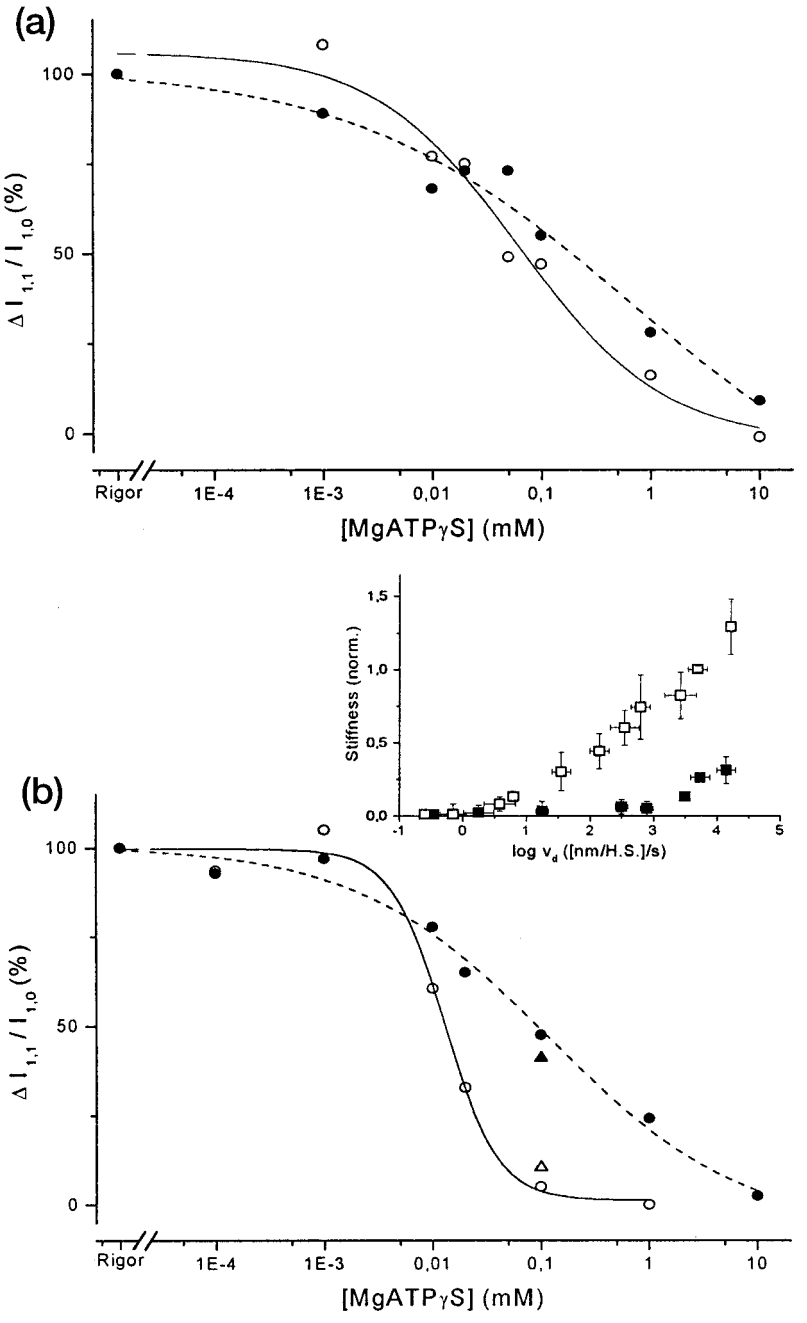

FIGURE 3 Change in intensity ratio of the two most intense equatorial reflections ($I_{1,1}/I_{1,0}$) at increasing concentrations of MgATPγS. Change normalized to difference between $I_{1,1}/I_{1,0}$ in rigor and $I_{1,1}/I_{1,0}$ under relaxing conditions. (*a*) Natural fiber bundle (bundle size: 0.7 × 0.2 mm) at pCa 4.5 (●) and at pCa 8.0 (○); ionic strength = 80 mM. (*b*) Array of 30 single fibers at pCa 4.5 (ionic strength 170 mM, ●; ionic strength 80 mM, ▲) and at pCa 8.0 (ionic strength 170 mM, ○; ionic strength 80 mM, △). Rigor = MgRigor. T = 1°C. Representative data are from one natural bundle or from one single fiber array, respectively. Sigmoidal curves were fitted to the data points. The diffraction patterns for *a* were recorded at the laboratory x-ray generator. (*Inset*) (modified from Kraft et al., 1992) The effect of calcium on the relation between fiber stiffness and speed of stretch in the presence of 10 mM MgATPγS. T = −3°C, ionic strength = 75 mM. ■, pCa 8; □, pCa 4.5; n = 4–7 fibers ± SEM. Values are normalized to fiber stiffness at the second fastest stretch at pCa 4.5.

the overall intensity in rigor at low calcium, because according to diffraction theory and our own preliminary tests, for the limited detecting area, total integrated intensity scattered by a muscle sample is the same for all conditions. Such normalization was necessary to exclude effects that could arise from changes in muscle mass in the beam at the different conditions. Nevertheless, the results from both synchrotron sources were the same; the data shown in Fig. 8 are from the experiments in Daresbury.

Data analysis

Data analysis of the MgATPγS series from the DESY was carried out on a Silicon Graphics INDIGO workstation (Mountain View, CA), using a program modified by D. Gilroy (National Institutes of Health, Bethesda, MD) from the software Profida, originally written by M. Lorenz (Max-Planck-Institut, Heidelberg, Germany). Detailed features of the program are described by Xu et al. (1997). The data recorded at different ionic strengths were analyzed on a PC using the programs STAFO and SCACO (provided by G. Rapp, EMBL Outstation, Hamburg, Germany) and OTOKO (Koch and Bendall, 1981; Boulin et al., 1986). The two programs had essentially the same features, and analysis with them followed a similar routine. The data recorded at the SRS were analyzed on a Silicon Graphics ONYX workstation (RRZN, University of Hannover) with the programs "bsl" and XOTOKO of the CCP13 software package provided by the SRS. To determine the intensity profile of the 59-Å layer line or the intensity of meridional reflections, a Gaussian function was fitted to the respective peaks of a series of sections (see below) after the background under the peaks had been subtracted using the software ORIGIN (Microcal, Northhampton, MA, USA).

The general anaysis procedure was as follows. After linearization of the imaging plate data or correction of the detector data for nonlinearities of

the detector, the diffraction patterns were normalized by the output of an ionization chamber that reflected the beam intensity integrated over exposure time. Then the patterns were rotated and shifted so that the center of the patterns was at the origin of the Cartesian coordinates. This allowed us to add patterns together, which were recorded under identical conditions with different single-fiber arrays. To reduce experimental error, a complete series of all different experimental conditions was recorded from each single fiber array. After subtraction of the machine background, the patterns recorded at the SRS were normalized to the overall intensity of the rigor pattern as described above. Finally, the added patterns were folded, and profiles in the horizontal and vertical directions were obtained for further analysis (see figure legends for the width of the integrated areas). The profiles of the actin layer line at 59 Å were obtained by integrating the intensity of the layer line in a series of 22 (Fig. 8) or 35 (Fig. 12) vertical sections parallel to the meridian, which covers essentially the area from the meridian to the edge of the pattern. Subsequently, these intensities were plotted as a function of the radial position of the respective section, and the data in Fig. 8 and Fig. 12 were smoothed by a five-point adjacent averaging procedure (ORIGIN, Microcal).

The spacings of the reflections were calibrated as described by Xu et al. (1997). For reference, the meridional reflection at 1/144.3 $Å^{-1}$ under rigor conditions at 170 mM ionic strength and 20°C was used. However, for simplification we shall call the third meridional myosin reflection the 143-Å reflection and the group of reflections around the second meridional reflection the 215-Å reflection, despite the differences in their spacing under various conditions.

To get an estimate of the statistical significance of the results, we determined the counting error for the data we obtained in Daresbury. The total number of counts (without background) in an intensity profile of the actin layer line at 59 Å, for example, was determined in the original, untreated individual patterns. For the shortest exposure times, the values were ~55,000 counts in the detector recordings. Taking into account that the analyzed patterns were the sum of four to seven individual patterns and that these patterns were folded to improve the signal-to-noise ratio, the counting error in the final data is ≤1%.

The results presented in this paper are from one set of data obtained during one synchrotron run. However, we want to mention that we repeated both the MgATPγS experiments and the experiments at different ionic strengths twice during different synchrotron runs. The results of all data sets are fully consistent with the data we show in this paper.

Confocal microscopy

Diffusion of rhodamine-phalloidin as well as of an anti-myomesin antibody (provided by T. Wallimann, Federal Institute of Technology, Zürich, Switzerland) and a secondary, fluorescein isothiocyanate-labeled antibody into an array of skinned single fibers versus diffusion into a natural fiber bundle was studied by confocal microscopy according to a method described earlier (Kraft et al., 1995). All diffusions occurred under relaxing conditions. The single-fiber array was prepared as described above for the x-ray diffraction experiments. Small natural fiber bundles were isolated from psoas muscle by carefully removing the connective tissue between the naturally occurring muscle fiber bundles without any damage to the fibers (as described in Xu et al., 1997). The single-fiber arrays and the natural bundles, respectively, were mounted in a shallow (depth ~0.8 mm) flow-through chamber mounted on a microscope slide. The different molecules were diffused into the muscle before observation without covering the chamber in a drop (100 μl) of solution to ensure that the single-fiber array or the natural bundle was completely surrounded by solution. Furthermore, the antibody solution was stirred several times during the incubation. After incubation the specimens were rinsed with relaxing solution. For confocal image recording, the chamber was covered with a coverslip so that the solution could still be changed by sucking it through the chamber. The small chamber was mounted with a chamber holder on an inverted microscope (Axiovert; Zeiss, Germany), equilibration was recorded with a 20× water immersion objective (Zeiss) and a Biorad MRC600 confocal scanner.

RESULTS

To study structural features of weak cross-bridge interaction with the actin filament in its activated and nonactivated forms, a pure population of weak binding cross-bridge states must be ensured, even in the presence of calcium; i.e., a mixture of weak and strong binding states due to turnover in the presence of calcium must be avoided. For part of the study we therefore used MgATPγS as an analog for weak binding cross-bridge states in both the presence and absence of calcium (Kraft et al., 1992).

Control experiments, however, showed that with fiber bundles, even for the slowly hydrolyzable MgATPγS, diffusion seems to be too slow to prevent depletion of substrate and accumulation of products. This became evident when MgATPγS was titrated in natural fiber bundles (Fig. 3). The changes in equatorial reflections as the MgATPγS concentration was increased were not the same as the results obtained previously with single fibers (cf. Kraft et al., 1992). Even in small natural bundles (diameter ~0.7 mm × ~0.2 mm) the intensity ratio of the [1,1] and [1,0] equatorial reflections ($I_{1,1}/I_{1,0}$; Fig. 3 *a*) in the absence of calcium could almost not be distinguished from that obtained in the presence of calcium. This suggested that the thin filaments were activated, even in the absence of calcium, presumably by nucleotide-free cross-bridges or cross-bridges with ADP in the active site. We therefore had to develop a new technique that allows recording of 2D x-ray diffraction patterns from single skinned muscle fibers with sufficient intensity for weak reflections like actin and myosin layer lines. To obtain sufficient intensity, we mounted 30 isolated single fibers side by side in our setup, forming a single-fiber array. Repeating the ATPγS titration in the presence and absence of calcium with our array of 30 single fibers, the observed titration curve showed the same difference in nucleotide concentration dependence between the presence and absence of calcium as previously recorded with individual single fibers (Fig. 3 *b*). In particular, in the absence of calcium there was a decrease in the equatorial intensity ratio ($I_{1,1}/I_{1,0}$), indicating binding of MgATPγS to the cross-bridges, at the same low nucleotide concentrations as in our previous single-fiber work (Kraft el al., 1992), that is, at lower nucleotide concentrations than observed with natural fiber bundles. This indicates that even the slow hydrolysis of MgATPγS (Bagshaw et al., 1972) is sufficient to result in depletion of MgATPγS and accumulation of products in the natural fiber bundles, specifically because no backup system for regeneration of MgATPγS from MgATP can be applied.

Confocal microscopy studies (Fig. 4) demonstrated that diffusion properties in single-fiber arrays and in natural fiber bundles are indeed different. For these control experiments we used small natural fiber bundles and single-fiber arrays of similar size that were prepared as for the x-ray experiments. We studied diffusion of rhodamine-phalloidin, a molecule of molecular weight similar to that of nucleotides, and diffusion of a fluorescently labeled antibody. It

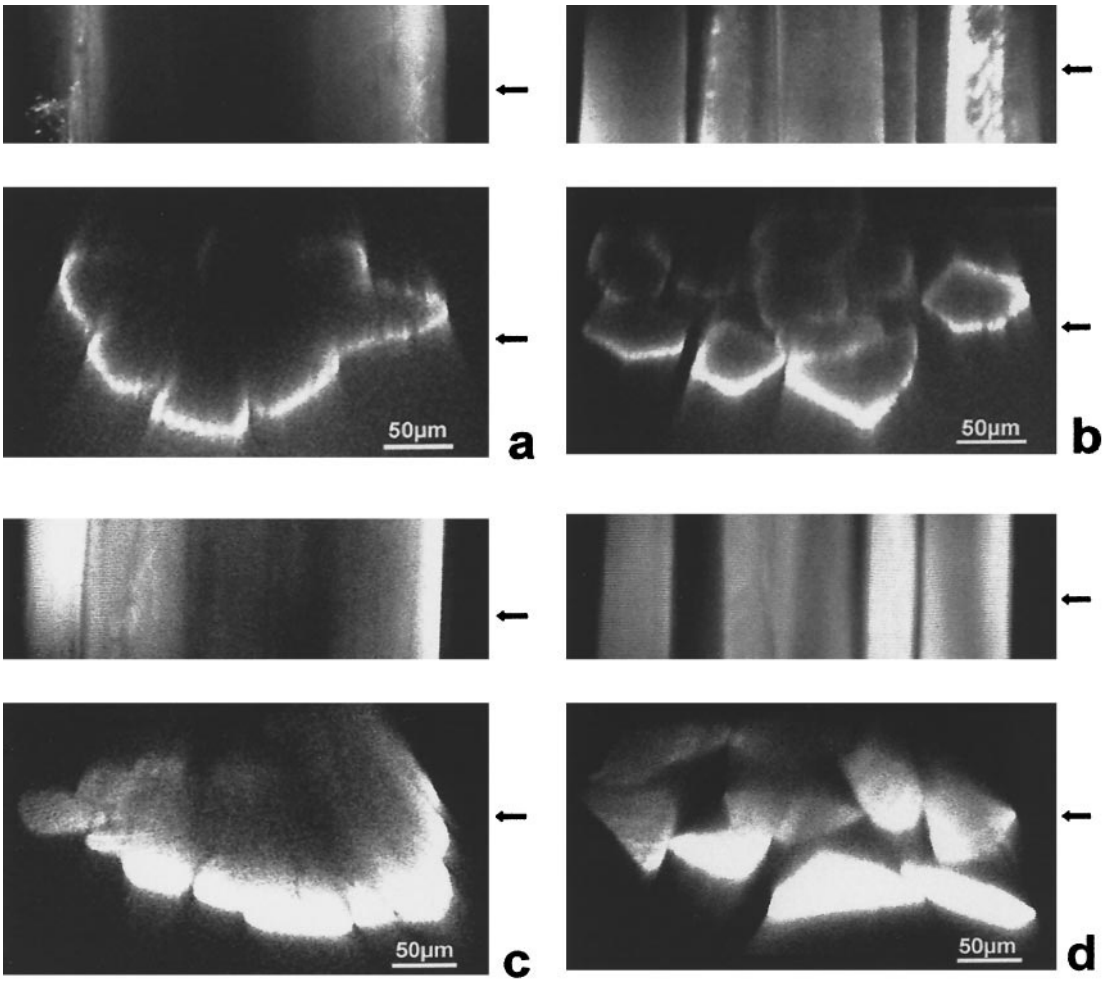

FIGURE 4 Optical sections through small natural bundles of muscle fibers (*left panels*) and groups of 10 single fibers (*right panels*). (*a, b*) After 5 h of incubation in relaxing solution with a primary antibody against the M-line protein myomesin and 20 min of incubation in relaxing solution containing a secondary, FITC-labeled antibody. (*c, d*) After 2 min of incubation in relaxing solution with 10 μM rhodamine-phalloidin. $T = 5°C$, ionic strength = 170 mM. (*Upper panel in a, b, c, d*) Longitudinal sections at the positions indicated by the arrows next to the lower images. (*Lower panel in a, b, c, d*) Cross sections (*Z* scans) at the positions indicated by the arrows next to the upper images. The loss of intensity toward the upper parts of the cross sections is the result of scatter of the exciting and emitted light and of increasing focus size due to increasing aberrations in thick specimens (~200 μm).

was found that equilibration of natural fiber bundles (Fig. 4, *a* and *c*) with either Rh-phalloidin or with the antibodies occurred mainly by diffusion of the fluorescently labeled molecules from the outer border of the whole bundle toward the center. Only a minor part of the diffusion into the fibers within the bundle was from molecules that had penetrated the space between the fibers. In these natural bundles the fibers are quite densly packed, so that diffusion between the fibers is rather limited; that is, the whole bundle essentially behaves as a single homogeneous system. In contrast, once the fibers are first isolated and then mounted in our array of parallel single fibers, the gaps between the fibers are much larger. Diffusion in such an array of single fibers is directly from the solution surrounding each fiber (Fig. 4, *b* and *d*). As shown previously (Kraft et al., 1995b), diffusion of the significantly smaller Rh-phalloidin molecule occurred on a much faster time scale than diffusion of the antibodies. For both fluorescently labeled molecules, the time for equilibration of all fibers within an array of single fibers appears equivalent to the time for equilibration of a single fiber. Note that in Fig. 4 (*lower panels*) with increasing depth in the *Z* scans, the fluorescence intensity decreases. This is due to 1) scatter of exciting and emitted light and 2) increasing aberrations resulting in increasing focus size. Nevertheless, both *Z* and longitudinal sections demonstrate that a natural fiber bundle shows fluorescent labeling like one large fiber, whereas in our single-fiber arrays each fiber is labeled as an individual fiber.

Weak cross-bridge binding to actin in the presence and absence of calcium

Previously we showed that cross-bridges with MgATPγS as a nucleotide analog exhibit features that allow us to classify them as weak binding states of the myosin head (Kraft et al.,

1992). The observed difference in detachment and attachment rate constants of the cross-bridges with MgATPγS (Fig. 3 *b*, *inset*; Kraft et al., 1992) suggested that in the presence of calcium, that is, when the actin filaments are activated, weakly binding cross-bridges interact differently with the actin filament. To see whether this difference in actin interaction is associated with a different structure of the actin-myosin complex, we recorded 2D x-ray diffraction patterns under the same conditions as in the mechanical experiments, that is, with 10 mM MgATPγS at 1°C. The highest possible nucleotide concentration and very low temperature had previously been shown to be necessary to provide nucleotide saturation not only at low but especially at high calcium concentrations (Kraft et al., 1992). To ensure that a rather large fraction of cross-bridges with MgATPγS is attached to actin, the ionic strength was set at 80 mM, the lowest level possible with 10 mM MgATPγS.

Fig. 5, *a* and *b*, shows 2D x-ray diffraction patterns of single-fiber arrays recorded in the presence of 10 mM MgATPγS at low (Fig. 5 *a*) and high (Fig. 5 *b*) calcium concentrations. For comparison, Fig. 5 *c* shows the 2D x-ray diffraction pattern recorded in the presence of 10 mM MgATP under otherwise identical conditions (without hexokinase and Ap_5A). All three patterns are very similar and predominantly show features that are typical for relaxed rabbit skeletal muscle fibers at low temperature such as myosin-based layer lines, a strong 215-Å reflection, and actin features (e.g., 59-Å actin layer line) that are not very strong. As an example for conditions where cross-bridges are in a strong binding conformation, in Fig. 5 *d* a diffraction pattern recorded under nucleotide-free conditions (magnesium rigor) at high calcium concentration is shown. In contrast to the diffraction patterns recorded with MgATPγS or with MgATP, the rigor pattern is dominated by actin-based layer lines, whereas the myosin layer lines are hardly detectable.

For a more detailed analysis of structural changes between the different states, we determined the intensity profiles of meridional reflections (Fig. 6), as well as profiles of myosin- and actin-based layer lines of the 2D x-ray diffraction patterns (Figs. 7 and 8). Fig. 6 shows the meridional profile (Fig. 6 *a*) and the individual meridional reflections

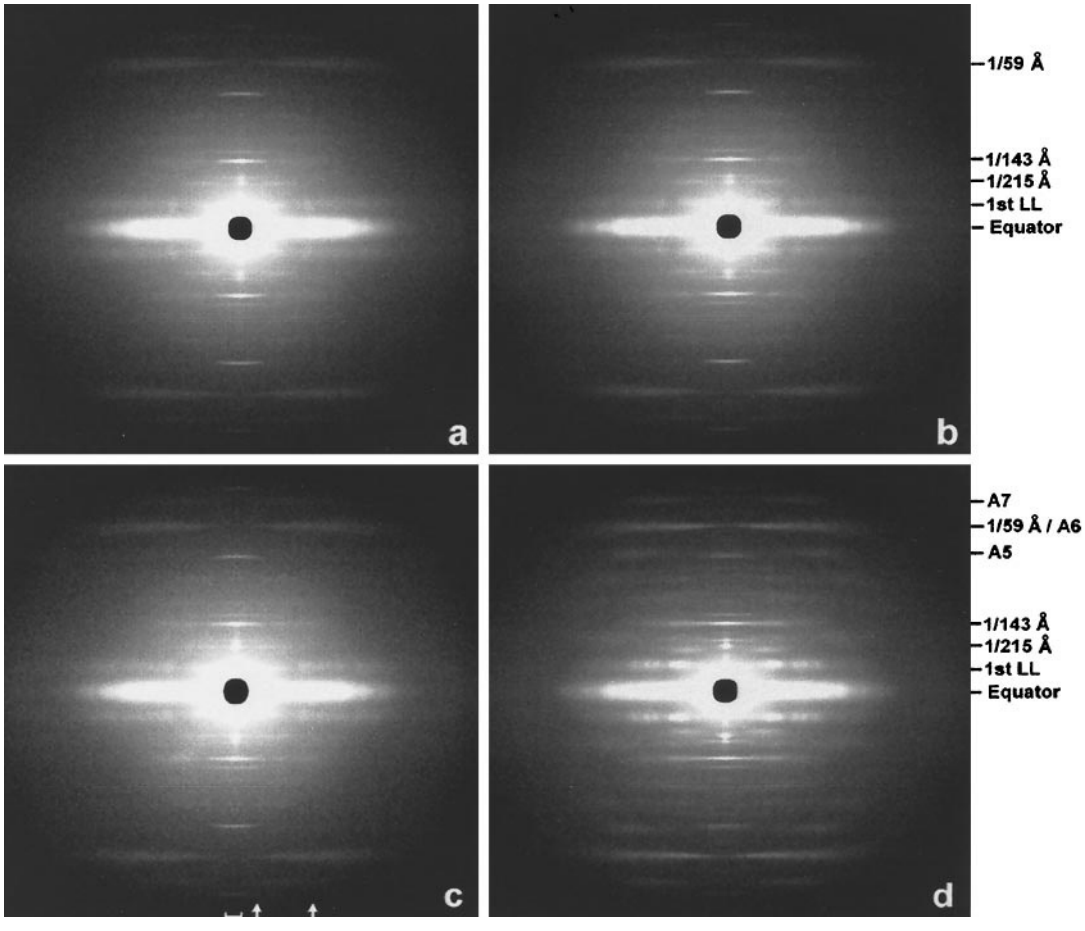

FIGURE 5 Two-dimensional x-ray diffraction patterns of single fiber arrays in the presence of 10 mM MgATPγS, (*a*) in the absence of calcium (pCa 8.0), (*b*) in the presence of calcium (pCa 4.5), (*c*) in the presence of 10 mM MgATP (pCa 8.0), and (*d*) in the absence of nucleotide (magnesium rigor). $T = 1°C$, ionic strength = 80 mM. Patterns were recorded from the same fiber arrays for all conditions ($n = 4$ single fiber arrays). 1st LL indicates the combined first actin and first myosin layer lines. A5, A6, A7 are actin layer lines. The horizontal bar in *c* marks the width of the area integrated for the meridional profiles in Fig. 6. The arrows in *c* mark the area that was horizontally integrated for the layer line profiles shown in Fig. 7.

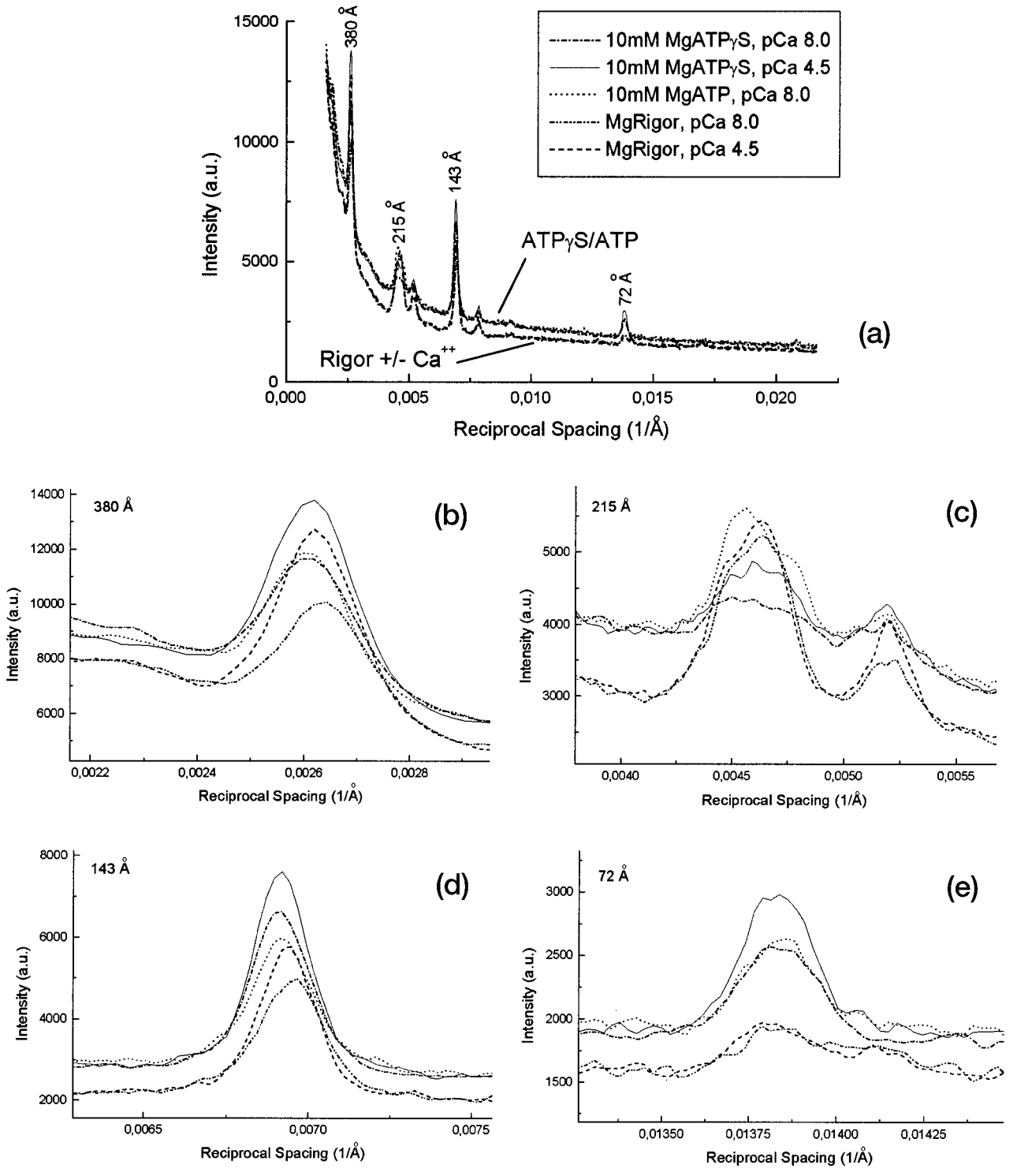

FIGURE 6 Profiles of meridional reflections of the diffraction patterns recorded in the presence of 10 mM MgATPγS (pCa 8.0 and pCa 4.5), in the presence of 10 mM MgATP (pCa 8.0), and in the absence of nucleotide (magnesium rigor; pCa 4.5 and pCa 8.0). Ionic strength = 80 mM, $T = 1°C$. The width of the integrated meridional area was determined by the width of the 215-Å reflection (of Fig. 5). (*a*) Meridional profile. The upper line consists of both MgATPγS profiles (pCa 8 and pCa 4.5) and the MgATP profile; the lower line consists of both rigor profiles (pCa 8 and pCa 4.5). (*b*) Reflection at 380 Å. (*c*) Reflection at 215 Å. (*d*) Reflection at 143 Å. (*e*) Reflection at 72 Å. The difference in vertical position of the reflections in rigor is due to the lower background under these conditions (Xu et al., 1997).

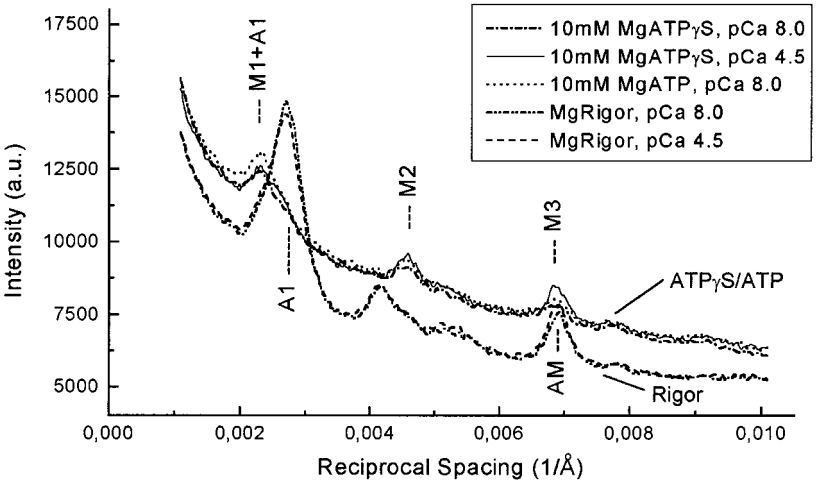

FIGURE 7 Integrated intensities of the myosin and actin layer lines of diffraction patterns recorded in the presence of 10 mM MgATPγS (pCa 8.0 and pCa 4.5) and 10 mM MgATP (pCa 8.0) and in the absence of nucleotide (magnesium rigor; pCa 4.5 and pCa 8.0). Ionic strength = 80 mM, T = 1°C. The profiles are from the patterns shown in Fig. 5. The myosin layer line profiles were obtained by horizontally integrating the area in the folded patterns, which is indicated by the two arrows in Fig. 5 *c*. M1 + A1 = combined first actin and first myosin layer line. M2, M3 = myosin layer lines. A1 = actin layer line.

(Fig. 6 *b–e*) at 380 Å, 215 Å, 143 Å, and 72 Å, the intensities of which were integrated. Most obviously, Fig. 6 *a* shows a clear difference in background intensity between rigor profiles and profiles obtained under relaxing conditions. In the presence of MgATP or MgATPγS the background is significantly higher than in rigor, as shown previously (Xu et al., 1997).

In agreement with earlier studies (Rome et al., 1973), the troponin-based meridional reflection at 380 Å was found to be sensitive to calcium. Fig. 6 *b* shows that the integrated intensity of the 380-Å reflection (I_{380}) increases significantly upon calcium binding to troponin C. This holds true not only for conditions with MgATPγS (i.e., in the presence of weakly binding cross-bridges), but also for rigor conditions. The profile for 10 mM MgATP in Fig. 6 *b* essentially overlays the profile for MgATPγS without calcium.

The myosin-based reflection at 215 Å (Fig. 6 *c*) seems to be quite sensitive to the type and concentration of nucleotide used. The data in Fig. 6 *c* show that I_{215} is rather high under rigor conditions with and without calcium. At 10 mM MgATP, I_{215} is much lower (68% of rigor at pCa 8), and with 10 mM MgATPγS in the absence of calcium I_{215} is smallest (28% of rigor at pCa 8). This latter difference might be partially due to the mainly MgATP-like state that is induced with MgATPγS instead of the MgADP · P_i state, which is thought to predominate when MgATP is present (Goody et al., 1975). Nevertheless, I_{215} increases somewhat when calcium is added in the presence of MgATPγS (40% of rigor at pCa 8). For comparison, I_{215} in rigor is still smaller than in the presence of only 1 mM MgATP (I_{215} in rigor is 78% of I_{215} under relaxing conditions with 1 mM MgATP at 80 mM ionic strength, 1°C; cf. Fig. 10). This is in agreement with previous studies (Xu et al., 1997).

The integrated intensity of the reflection around 143 Å (Fig. 6 *d*) is stronger with 10 mM MgATPγS at pCa 8 than with 10 mM MgATP (by 20%) and stronger than in rigor at low calcium (by 23%) and at high calcium (by 18%). I_{143} increases with calcium in the presence of MgATPγS (by 12%) compared to MgATPγS, pCa 8. It should be noted, however, that I_{143} in rigor (pCa 8) is still stronger (by 87%) than under relaxing conditions with only 1 mM MgATP (80 mM ionic strength, 1°C; cf. Fig. 10). The fact that with MgATPγS the 143-Å reflection remains at the same position as with MgATP, whereas in rigor it shifts to a slightly smaller spacing, can be taken as evidence that MgATPγS cross-bridges do not change toward a rigor-like attachment, even when calcium is raised.

The meridional myosin reflection at 72 Å (Fig. 6 *e*) shows very similar intensity with 10 mM MgATP and with MgATPγS in the absence of calcium, but again it is much higher when calcium is raised in the presence of MgATPγS. The reflection becomes rather weak under rigor conditions, where it has the same intensity with and without calcium.

The integrated intensities of the second and third myosin layer lines (M2, M3; Fig. 7) seem to increase somewhat with the addition of calcium in the presence of MgATPγS.

We also obtained intensity profiles of the actin layer line at 59 Å (Fig. 8), which is expected to be sensitive, for example, to cross-bridge attachment and to calcium binding to the thin filament. The profiles were reconstructed from the integrated intensities in 22 neighboring sections cutting across the 59-Å layer line. Fig. 8 shows that the integrated intensities of the 59-Å layer line (I_{59}) increased at high calcium in the presence of MgATPγS compared to low calcium, yet without a change in intensity distribution. Integration of both profiles indicated an intensity increase of ~15%. Under nucleotide-free conditions (magnesium rigor), I_{59} was much higher and the intensity maximum was shifted much closer to the meridian, as has been shown previously (Huxley and Brown, 1967). Interestingly, the addition of calcium caused an increase in I_{59} in rigor, too.

Thus the question arises whether the intensity increase in I_{59} with calcium in the presence of MgATPγS is due to a change in the attachment mode of cross-bridges to activated thin filaments, resulting in additional labeling of the actin helix, or whether it is induced merely by calcium binding to regulatory proteins (cf. Kress et al., 1986; Yagi and Matsubara, 1989). We therefore recorded 2D x-ray diffraction

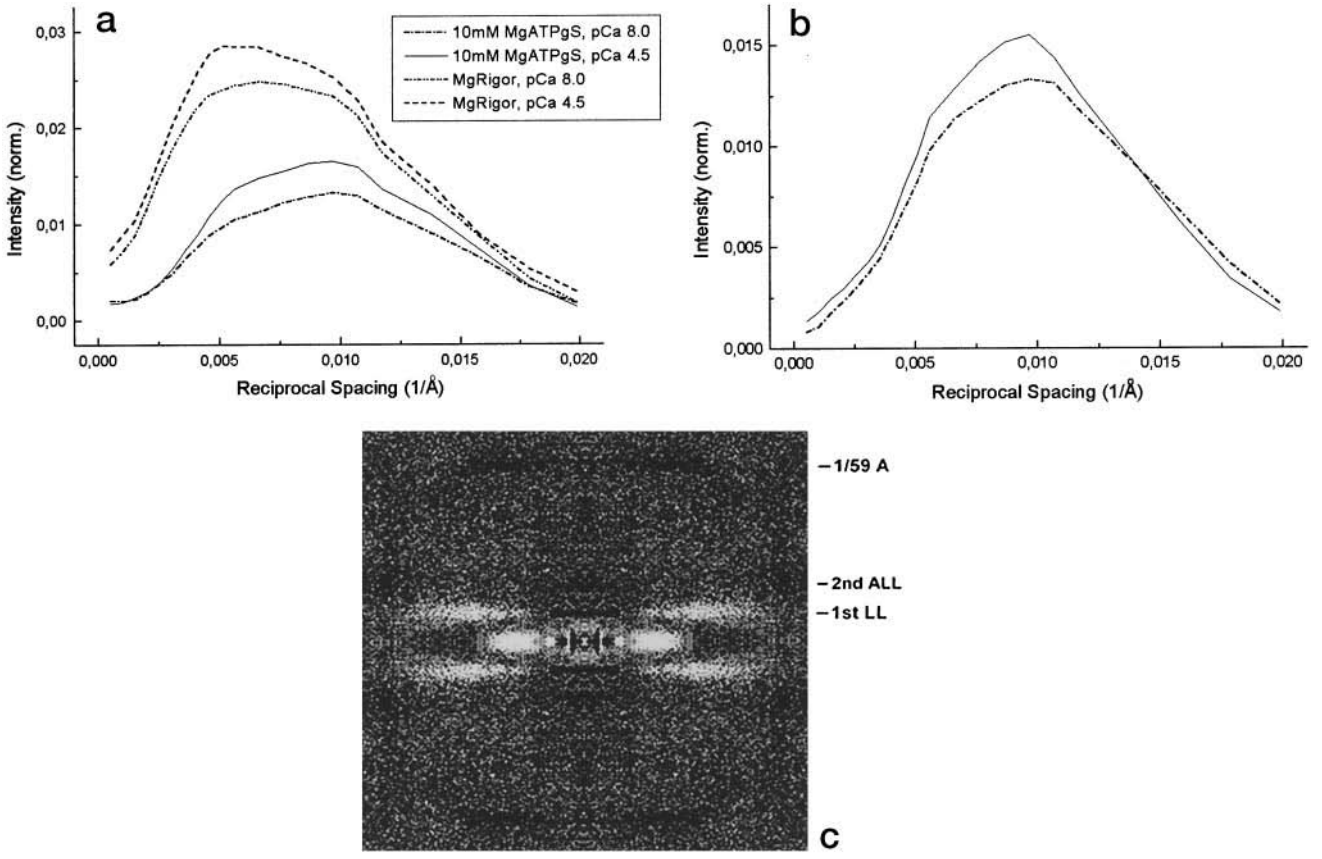

FIGURE 8 Intensity profiles along the actin layer line at 59 Å. (*a*) At normal sarcomere length (2.4 μm) in the presence of 10 mM MgATPγS (pCa 8.0 and pCa 4.5) and in the absence of nucleotide (magnesium rigor; pCa 4.5 and pCa 8.0) from the patterns in Fig. 5. (*b*) At a sarcomere length of ~4.0 μm in the presence of 10 mM MgATPγS at pCa 8.0 and pCa 4.5. (*c*) Two-dimensional difference pattern of diffraction patterns recorded at pCa 8.0 and pCa 4.5 in the presence of 10 mM MgATPγS at a sarcomere length of ~4.0 μm. Ionic strength = 80 mM, T = 1°C. The profiles in *a* and *b* were obtained by integrating the layer line intensity in 22 neighboring sections parallel to the meridan and subsequent plotting of the layer line intensity in each section versus radial position of the section. In *c* differences in intensities are indicated by changes in black pixel density. An increase in pixel density above average indicates an intensity increase at pCa 4.5. Lower pixel density (*white areas*) indicates decreased intensity at pCa 4.5 relative to pCa 8.0. Data in *b* and *c* are from seven single-fiber arrays.

patterns with MgATPγS at high and low calcium concentrations from single-fiber arrays that were stretched to very long sarcomere length (4 μm) so that cross-bridge attachment would be minimized (Fig. 8, *b* and *c*). Analysis of the 59-Å actin layer line again showed an increase in I_{59} at high calcium compared to low calcium. I_{59} increased by ~7%, which is half of the increase found for fibers at normal sarcomere length. This suggests that a good part of the increase in I_{59} seems to be due to calcium binding to the regulatory proteins. Yet it cannot be ruled out that some of the increase might also be attributed to a different attachment mode of weakly binding cross-bridges in the presence of calcium. The difference pattern of the 2D diffraction patterns recorded at very long sarcomere length with MgATPγS at low and high calcium in Fig. 8 *c* also shows the increase in I_{59}. It is indicated in this representation as an accumulation of black pixels. In addition, the difference pattern also shows a decrease in pixel density at the position of the first actin layer line, indicating an intensity decrease of the first actin layer line, and just at the very edge of the pattern, also an intensity increase (increased pixel density) of the second actin layer line upon addition of calcium. Both of these changes have been described earlier (Huxley, 1972; Haselgrove, 1972; Vibert et al., 1972; Kress et al., 1986; Yagi and Matsubara, 1989; Wakabayashi et al., 1991, 1993).

Weak cross-bridge binding to actin at <50 mM ionic strength

To overcome the problem that cross-bridges with MgATPγS might not represent a pre-force-generating state of the M · ADP · P_i/AM · ADP · P_i type, we made use of the proposal that lowering the ionic strength to <50 mM induces the same transition in the state of the thin filament as does raising calcium at ionic strength > 50 mM (Head et al., 1995). On this basis, recording 2D patterns at 80 mM and 30 mM ionic strength at low calcium should allow us to study possible changes in the actin attachment of the weak

binding cross-bridge states in the presence of MgATP upon the activation of the actin filament, just as with the addition of calcium.

The advantages of this approach compared to the MgATPγS experiments are 1) The lower ionic strength makes it possible to study a larger fraction of weakly attached cross-bridges (Brenner et al., 1986). 2) It makes it possible to use not only 1°C but also 20°C as the experimental temperature, which is closer to physiological conditions. 3) Some shift of the distribution of the cross-bridges from M · ATP/AM · ATP states (at 1°C) toward a larger fraction of M · ADP · P_i/AM · ADP · P_i states (at 20°C) was proposed to occur (in myofibrils, however, at both low and high temperatures, the predominant intermediates were found to be the M · ADP · P_i/AM · ADP · P_i states; Ma and Taylor, 1994). Therefore, this approach allows us to study at 20°C effects on mostly the ADP · P_i states instead of the ATP-like states that predominate in the presence of MgATPγS (Goody et al., 1975).

Again, to avoid MgATP depletion due to some MgATP turnover, particularly at an ionic strength of 30 mM and 20°C, these patterns required recording with single-fiber arrays instead of natural fiber bundles. As for the MgATPγS experiments, 30 single fibers were mounted together for each experiment. Unfortunately, to do the same experiment as a control with MgATPγS was not possible, because for an ionic strength as low as 30 mM, only 1 mM MgATPγS can be used, which, however, was found to be insufficient for saturation, even at low temperature (Kraft et al., 1992).

Fig. 9 shows the 2D x-ray diffraction patterns recorded under relaxing conditions at 30 mM and 80 mM ionic strength at 1°C (Fig. 9, *a* and *b*) and 20°C (Fig. 9, *c* and *d*). Analysis of equatorial and meridional reflections as well as actin and myosin layer lines confirms that the patterns at both ionic strengths and both temperatures exhibit features of weak binding cross-bridge states (Xu et al., 1997; Malinchik et al., 1997). The intensity ratio of the equatorial reflections ($I_{1,1}/I_{1,0}$) increases with lower ionic strength, that is, with an increase in the fraction of attached myosin heads, as has been shown earlier (Brenner et al., 1982). Under relaxing conditions at 1°C and 80 mM ionic strength, $I_{1,1}/I_{1,0}$ is 49% of rigor; at 1°C and 30 mM ionic strength it is

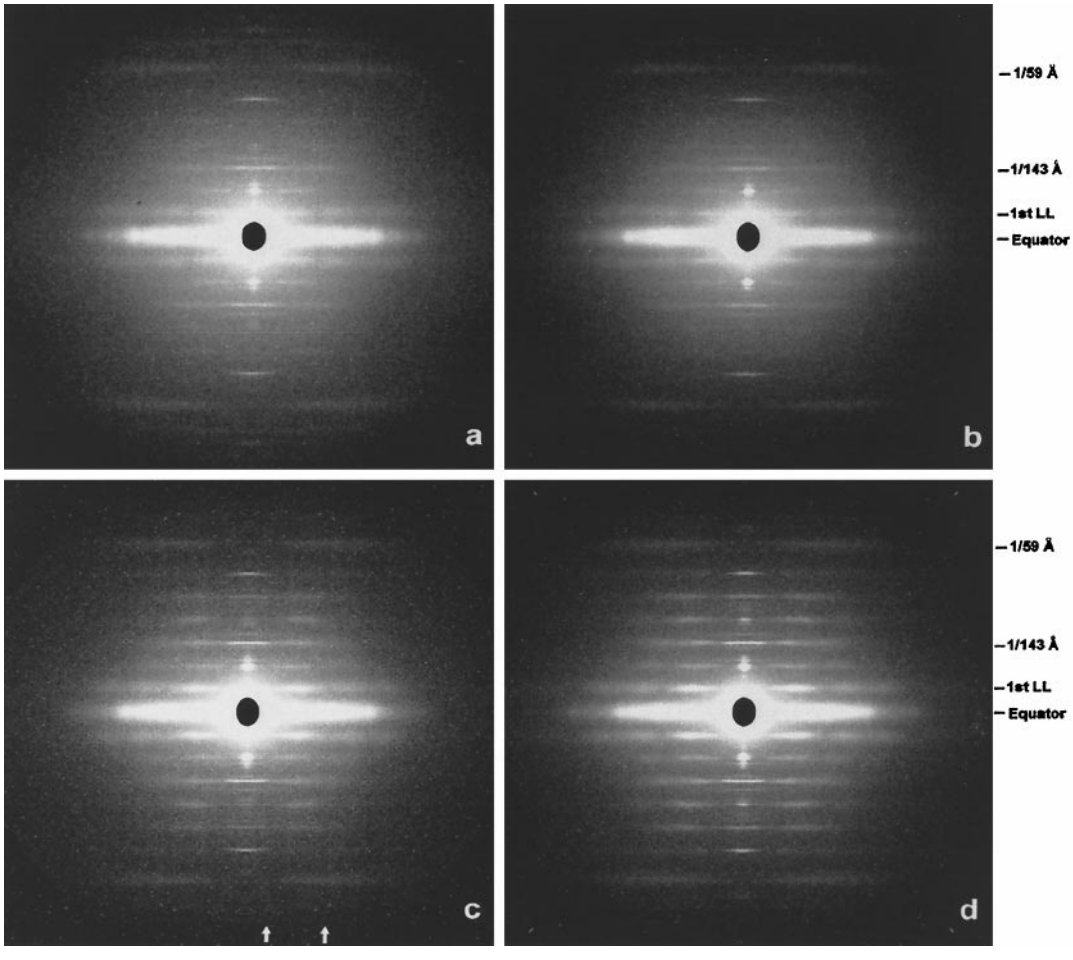

FIGURE 9 Two-dimensional x-ray diffraction patterns of single-fiber arrays recorded under relaxing conditions at (*a*) 30 mM ionic strength, $T = 1°C$; (*b*) 80 mM ionic strength, $T = 1°C$; (*c*) 30 mM ionic strength, $T = 20°C$; (*d*) 80 mM ionic strength, $T = 20°C$. All patterns were recorded from the same single fiber arrays ($n = 5$ single fiber arrays). 1st LL indicates the combined first actin and first myosin layer lines. The arrows in *c* indicate the area that was horizontally integrated for the layer line profiles shown in Fig. 11.

68% of rigor. Both values are consistent with our previous results from single fibers (Kraft et al., 1992). At 20°C, $I_{1,1}/I_{1,0}$ under relaxing conditions at 80 mM and at 30 mM ionic strength is 18% and 31%, respectively, of the ratio in rigor. The meridional reflections change only very little when the ionic strength is reduced at 1°C or at 20°C (Fig. 10).

The main question, however, was whether lowering the ionic strength to <50 mM would induce an activation of the thin filament and as a consequence would result in a change in attachment mode of weakly bound cross-bridges, presumably into a stereospecific conformation, for example, as in rigor or during isometric contraction. In Fig. 10 the changes in the intensities of the meridional reflections when the ionic strength is lowered at 1°C and at 20°C under relaxing conditions can be compared with the changes of the profiles when going from relaxed to rigor. From these data it is obvious that the changes occurring as the ionic strength is lowered do not even qualitatively correspond to changes when a transition to stereospecific rigor-attachment occurs. For example, at 1°C the integrated intensity of the reflection at 143 Å is high in rigor and low in relaxation (I_{143} in relaxing solution at 1°C is 43% and 54% of rigor at 80 mM and 30 mM ionic strength, respectively). The reflection at 215 Å is strong, especially at 20°C in relaxation, and weak in rigor (I_{215} in rigor at 20°C is 30% and 35% of relaxing conditions at 80 mM and 30 mM ionic strength, respectively).

All of the layer line profiles shown in Fig. 11 are dominated by myosin layer lines, in contrast to profiles obtained under rigor conditions (cf. Fig. 7). At 1°C, the intensity of the myosin layer lines decreases with increasing ionic strength (at 80 mM ionic strength, M1 + A1 (combined first layer line), M2, and M3 are 61%, 81%, and 94%, respectively, of their integrated intensities at 30 mM ionic strength), which is consistent with earlier observations on rabbit muscle (Lowy et al., 1991; Xu et al., 1997). At 20°C, where myosin layer lines are much stronger, the intensity of the myosin layer lines seems to increase at 80 mM ionic strength, compared with 30 mM (at 80 mM ionic strength, M1 + A1, M2, and M3 are, respectively, 117%, 120%, and 138% of their intensity at 30 mM ionic strength), and the layer lines seem to become more diffuse at lower ionic strength.

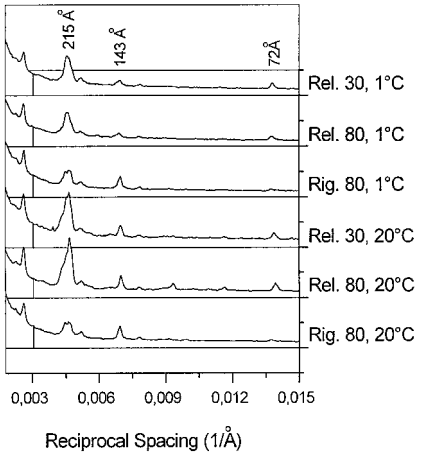

FIGURE 10 Meridional profiles of the diffraction patterns recorded (*from top to bottom*) under relaxing conditions at 30 mM ionic strength, 1°C and at 80 mM ionic strength, 1°C; in rigor at 80 mM ionic strength, 1°C; under relaxing conditions at 30 mM ionic strength, 20°C, and at 80 mM ionic strength, 20°C; and in rigor at 80 mM ionic strength, 20°C. The relaxed profiles are from the patterns shown in Fig. 9; the rigor profiles are from the same single fibers. The width of the integrated meridional area was determined by the width of the 215-Å reflection in Fig. 9.

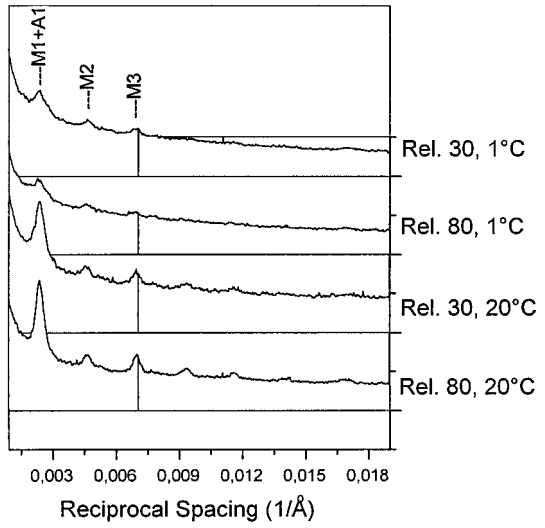

FIGURE 11 Profiles of the myosin layer lines recorded (*from top to bottom*) under relaxing conditions at 30 mM ionic strength, 1°C and at 80 mM ionic strength, 1°C; under relaxing conditions at 30 mM ionic strength, 20°C and at 80 mM ionic strength, 20°C. The profiles are from the patterns shown in Fig. 9 and were obtained by horizontally integrating the area in the folded patterns, which is indicated by the two arrows in Fig. 9 *c*.

Analysis of the intensity profile along the actin layer line at 59 Å when the ionic strength is lowered at 1°C and at 20°C (Fig. 12) shows that at both temperatures studied, I_{59} is independent of ionic strength. This is consistent with previous findings at different ionic strengths (Xu et al., 1987, 1997). It further confirms that as the ionic strength is lowered to <50 mM, no transition to a different attachment mode such as stereospecific attachment of weak binding cross-bridges occurs, because in this case an increase in I_{59} and/or a shift in intensity distribution would be expected. Furthermore, no evidence is found for a change in the 59-Å actin layer line, which would correspond to the effects seen with MgATPγS when the thin filament was activated by calcium.

Taken together, the results of the MgATPγS experiments and the analysis of equatorial and meridional reflections as well as actin and myosin layer lines reveal that 1) at either 10 mM MgATP or 10 mM MgATPγS at low calcium concentration, the intensity ratio of the equatorial reflections ($I_{1,1}/I_{1,0}$) and the intensities of most other reflections are very similar. Small differences can be observed for the meridional reflections at 215 Å and at 143 Å. 2) The addition of calcium in the presence of MgATPγS causes a

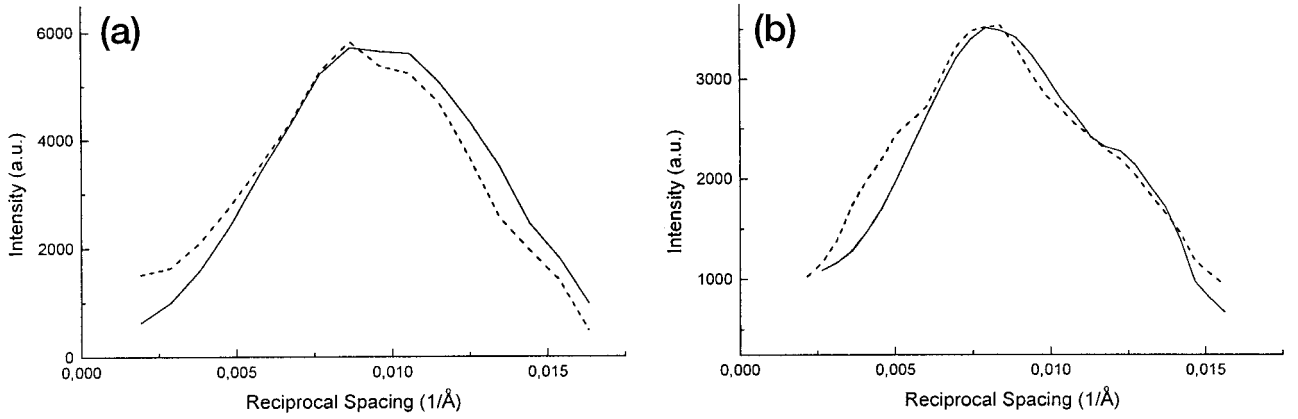

FIGURE 12 Intensity profiles along the actin layer line at 59 Å of the diffraction patterns shown in Fig. 9 recorded under relaxing conditions at 30 mM ionic strength (——) and at 80 mM ionic strength (– – –) at (*a*) $T = 1°C$ and (*b*) $T = 20°C$. The profiles were obtained from 35 neighboring sections, as described in Fig. 8.

small intensity increase in myosin-based meridional reflections, for example, at 143 Å and 72 Å. This increase is the opposite of the changes that occur when cross-bridges bind to actin in a rigor conformation. Furthermore, there was also no shift in the position of the 143-Å reflection in the presence of MgATPγS when calcium was added. 3) At least a good part of the observed increase of I_{59} upon addition of calcium can be attributed to the activation of the thin filament by calcium. The same holds true for intensity changes of the 380-Å meridional reflection and the first actin layer line. 4) Similar changes in the 380-Å meridional reflection, the first actin layer line, and the 59-Å actin layer line can also be observed with the addition of calcium under nucleotide-free (i.e., rigor) conditions.

The changes in the diffraction patterns observed when the ionic strength is lowered from 80 mM to 30 mM are also clearly different from the changes that occur when going from relaxed conditions into rigor. For example, there was no enhancement of the actin layer line at 59 Å, which could have indicated a transition to a different, actin-specific attachment mode at low ionic strength.

DISCUSSION

In this study we examined the effects of activation of the thin filament on the attachment mode of cross-bridges in weak binding states. Activation of the thin filament was achieved by adding calcium in the presence of MgATPγS, where the cross-bridges remain in the weak binding states. Another approach was to lower the ionic strength to <50 mM under relaxing conditions, which was proposed to have the same effect on the actin filament as raising calcium (Head et al., 1995).

When calcium is added in the presence of MgATPγS, subtle changes in the diffraction patterns are detectible that are different from changes that occur when the fibers are put into rigor. Lowering the ionic strength from 80 mM to 30 mM under relaxing conditions did not provide any evidence of changes in the diffraction patterns that would indicate a transition of the weakly attached cross-bridges to a distinctly different attached conformation.

Experimental requirements

In a previous study, Matsuda and Podolsky (1984) presented 2D x-ray diffraction patterns of relaxed rabbit skeletal muscle that showed features of weakly attached cross-bridges as well as features of rigor-like cross-bridges. Xu et al. (1987, 1997) demonstrated that these rigor-like features are not features of weakly attached cross-bridges in the presence of MgATP. Instead, rigor-like features in diffraction patterns from relaxed fibers seem to be due to slight MgATP turnover, which is sufficient to introduce nucleotide depletion. Such depletion can easily occur in the core of muscle bundles if the bundles are too large (Yu and Podolsky, 1990). As a result, nucleotide-free (strongly binding) cross-bridges form, which contribute to the resulting diffraction pattern and activate the fibers, thus resulting in a mixed, ill-defined diffraction pattern.

Here we show that for small natural muscle fiber bundles, even with MgATPγS, the nucleotide titration in the presence and absence of calcium is quite different from single fiber data (Fig. 3; Kraft et al., 1992). In small bundles, the nucleotide concentration dependence of $I_{1,1}/I_{1,0}$ is almost the same at pCa 8 and at pCa 4.5, whereas with single fibers there is a clear calcium effect. This is presumably due to a slight turnover of MgATPγS (Bagshaw et al., 1972) and rather slow diffusion within the bundles, resulting in nucleotide-free cross-bridges, which cause activation of the thin filament. Such starvation effects in bundles were also described previously by Goody and co-workers (1975). When studying MgATPγS in 2D x-ray diffraction experiments of small bundles of insect flight muscle, they had to use 20 mM MgATPγS to achieve complete saturation, even at low calcium (pCa 8). However, if such depletion problems occur even when the slowly hydrolyzable analog MgATPγS is

used or under relaxing conditions with MgATP, the problem must be even more serious under activating conditions, particularly at high temperature.

Therefore, in the present study we used arrays of single fibers to record the 2D x-ray diffraction patterns in the presence of MgATPγS at high and low calcium concentrations, and under relaxing conditions at ionic strengths below and above 50 mM, at which no high concentrations of creatine phosphate can be applied as a MgATP backup system. In addition, proper selection of experimental conditions like 10 mM MgATPγS and low temperature was necessary to overcome the much reduced affinity of the cross-bridges for MgATPγS, especially at high calcium concentration (Kraft et al., 1992).

Observed changes and their interpretation

Comparison between MgATP and MgATPγS at low calcium concentration

The 2D patterns recorded in the absence of calcium with 10 mM MgATP and with 10 mM MgATPγS, respectively, are very similar, as shown before in 2D x-ray diffraction of insect flight muscle (Barrington Leigh et al., 1972; Goody et al., 1975). This is consistent with our earlier studies, showing essentially identical fiber stiffness and stiffness-speed relations under both conditions (Kraft et al., 1992). One rather prominent difference is the intensity of the 215-Å meridional reflection. I_{215} is lowest in the presence of 10 mM MgATPγS, compared to rigor conditions and 10 mM MgATP. Because this reflection is even more prominent at 1 mM MgATP under conditions that are otherwise the same (Fig. 10), there seems to be an effect of the nucleotide concentration on I_{215}. Furthermore, the difference in I_{215} as well as the small differences in the intensities of other reflections between 10 mM MgATP and 10 mM MgATPγS might be due in part to the hydrolysis state of the nucleotide bound to the myosin head (ATPγS as an analog for ATP rather than for ADP · P_i states; Goody et al., 1975).

Comparison between MgATPγS at low and high calcium concentration and rigor

The changes in the diffraction patterns observed when calcium was added to fibers with saturating MgATPγS concentration can be attributed to two major effects: 1) calcium binding to the regulatory proteins and 2) possibly a slightly different conformation of the weakly attached heads.

The effects of calcium binding to the regulatory proteins is particularly evident in the difference pattern of the diffraction patterns with MgATPγS at low and high calcium concentration at very long sarcomere length (Fig. 8 *b*) and in the profile of the 380 Å meridional troponin reflection. Observed changes like the increase in I_{380}, as well as an intensity decrease in the first actin layer line and an intensity increase in the second actin layer line and the actin layer line at 59 Å, are consistent with the effects of calcium that have been described in the literature (Huxley, 1971; Haselgrove, 1972; Vibert et al., 1972; Rome et al., 1973; Yagi and Matsubara, 1989; Wakabayashi et al., 1991, 1993; Kress et al., 1986). Whereas intensity changes in the first and second actin layer lines can be attributed to tropomyosin movements (Parry and Squire, 1973), the changes in I_{59} were thought to be due to changes in troponin (Kress et al., 1986) or to subdomain movements of actin (Al-Khayat et al., 1995). Interestingly, the difference pattern of diffraction patterns recorded in rigor at low and high calcium (data not shown) reveals essentially the same changes as patterns recorded with saturating MgATPγS concentrations. This is shown in Fig. 6 *a* for the 380-Å meridional reflection and in Fig. 7 *b* for the actin layer line at 59 Å. This means that attachment of rigor cross-bridges (i.e., strongly binding cross-bridges, which are thought to activate the thin filament presumably by shifting tropomyosin) does not induce the same changes as calcium binding to troponin C.

In comparing the meridional profiles or the diffraction patterns recorded under relaxing conditions (with MgATPγS or with MgATP) with those recorded under rigor conditions, there is a very obvious difference in background intensity. As discussed previously (Xu et al., 1997), the higher background can be attributed to more disorder of the myosin heads under relaxing conditions than in rigor.

Evidence for a slightly different structure of the weakly binding MgATPγS cross-bridges at high calcium compared to low calcium may be derived from the observations that some meridional reflections (e.g., 143 Å, 72 Å) have higher intensity with calcium. These effects might indicate a change in axial orientation and/or myosin-based ordering of the myosin heads. An increase in the meridional I_{143}, for example, is thought to occur when myosin heads are oriented more perpendicular to the fiber axis (Barrington Leigh et al., 1972; Bordas et al., 1993; Lombardi et al., 1995). On the other hand, the fact that these changes are different from what happens when cross-bridges bind to actin in a rigor-like conformation (there myosin layer line intensities decrease, the 143-Å meridional reflection shifts to a different spacing, and I_{72} decreases) indicates that no significant part of the weakly binding cross-bridges assume a rigor-like stereospecific attachment mode when calcium is added. Further evidence that weak cross-bridge attachment at high calcium that is somewhat different from the weak cross-bridge attachment seen under relaxing conditions can be derived from the observation that I_{59} increases somewhat more as calcium is raised at normal sarcomere length compared to long sarcomere length. It was shown that weak cross-bridge attachment under relaxing conditions itself does not affect I_{59} at all, as shown at different ionic strength. The lack of a detectable shift of the layer line profile at 10 mM MgATPγS, pCa 4.5, toward the meridian, however, argues against any sizable part of the weakly attached heads assuming a stereospecific orientation with respect to the actin filament.

Yet the observed small changes in, e.g., meridional reflections and I_{59} with the addition of calcium in the presence

of MgATPγS could also be due to changes in the crystalline order between the different conditions. The diffraction pattern in Fig. 5 *b* seems to show a more pronounced crystallographic sampling along the myosin layer lines, particularly on the third myosin layer line compared to the pattern in Fig. 5 *a*, recorded at low calcium concentration. The difference in myosin binding to actin in the presence of MgATPγS at high calcium concentration (activated thin filament), which can be derived from the stiffness measurements (Fig. 3, *inset*) might result in a more regular arrangement of the myofilaments and therefore in better crystalline order. Yet this would still indicate that at high calcium the attachment of weakly binding cross-bridges to activated thin filaments is somewhat different from attachment to the inactive actin filament at low calcium.

Effects of reducing the ionic strength to less than 50 mM under relaxing conditions

Reducing the ionic strength to less than 50 mM under relaxing conditions was thought to have the same effect on the thin filament as the addition of calcium (Head et al., 1995). The question was whether lowering the ionic strength and thus activation of the thin filament would induce a transition of nonstereospecifically attached weakly binding cross-bridges to a different (e.g., stereospecifically attached) complex. Such a transition is expected, as mentioned in the previous paragraph, to cause an enhancement of actin layer lines and weakening of myosin-based features, e.g., changes qualitatively similar to the effects observed when changing to rigor.

However, 2D x-ray diffraction patterns recorded under relaxing conditions at 80 and 30 mM ionic strength provide no evidence for such a structural change in the weakly attached cross-bridges. This holds true not only for diffraction patterns recorded at low temperature (1°C), but also for patterns recorded at high temperature (20°C). As shown by others before (Wray et al., 1988; Wakabayashi et al., 1988; Lowy et al., 1991; Xu et al., 1997; Malinchik et al., 1997), at 20°C the myosin layer lines are much more prominent compared to 1°C, thus allowing a more sensitive detection of changes. Nevertheless, at each temperature the patterns recorded at the different ionic strengths appeared to be typical for relaxed muscle. The observed weakening of the myosin layer lines under relaxing conditions as the ionic strength is raised at 1°C (Fig. 11) is consistent with earlier findings for rabbit muscle, when ionic strength was raised from 20 mM (Lowy et al., 1991) or from 50 mM (Xu et al., 1997) to physiological values. At 20°C the myosin layer lines seem to become more diffuse at 30 mM ionic strength compared to 80 mM, and the integrated intensity decreases. A similar behavior was not clearly visible in our previous work (Xu et al., 1997), presumably because of the different ionic strength range studied. Reducing the ionic strength from 50 mM to 30 mM causes a quite significant further increase in weak cross-bridge attachment and might be the reason for the effects observed here. The increase in attachment is indicated by the changes in the equatorial intensities (Brenner et al., 1984) and the increase in fiber stiffness (Brenner et al., 1986), as well as by a shift of the centroid of the first layer line, as shown in a previous study (Xu et al., 1997). However, as in the case of MgATPγS, the observed changes in layer line intensities could also be attributed to changes in crystalline order at the different ionic strengths. This would mean that at higher ionic strength (80 mM), when fewer cross-bridges are weakly attached to actin, the system appears to be more ordered with better defined layer lines, particularly at 20°C. This is different from the effect of calcium activation in the presence of MgATPγS and might support the observation that lowering the ionic strength to lees than 50 mM for native thin filaments in our skinned fibers does not result in the same changes as induced by raising the calcium concentration.

Most importantly, the changes observed when the ionic strength is lowered are quite different from the changes that occur when going into rigor, and there is no evidence for enhancement of rigor-like features. For instance, neither at 1°C nor at 20°C was the 59 Å actin layer line intensity enhanced as the ionic strength was reduced to 30 mM, which is consistent with previous studies (Xu et al., 1987, 1997). Taken together, this leads to the conclusion that there is no evidence for structural changes of the weakly attached cross-bridges toward a more stereospecific type of attachment when ionic strength is lowered to $<$50 mM.

This conclusion is further supported by mechanical experiments showing no shift of the stiffness-speed relation of relaxed muscle fibers to lower speeds of stretch at 20 mM ionic strength compared to 80 mM ionic strength (Brenner et al., 1986). Such a shift, however, would be expected on the basis of the experiments with MgATPγS at low and high calcium concentrations and on the basis of stiffness measurements during unloaded isotonic shortening (Stehle et al., 1993), if lowering ionic strength would have the same effect on the actin filament as calcium activation. During shortening at near-maximum velocity the cross-bridges mainly occupy weak binding states, and the stiffness speed relation under these conditions was found to be essentially identical to that observed in the presence of MgATPγS with calcium but quite different from that seen under isometric conditions. In addition, one actually might expect that activation of the thin filament by lowering ionic strength in the presence of MgATP should activate the myosin ATPase, especially at 20°C, thus resulting in active cycling of the cross-bridges and force generation, which was not observed. This may indicate that, contrary to solution studies with reconstituted thin filaments (Head et al., 1995), the native actin filament in skeletal muscle fibers is not affected in the same way by changing ionic strength as it is by calcium activation.

Is the fraction of attached weakly binding cross-bridges too small for changes to be detected by 2D x-ray diffraction?

Because the observed changes with both experimental interventions are rather small, one might ask whether the

fraction of weakly attached cross-bridges that undergo structural changes is too small to be detected in 2D x-ray diffraction patterns.

As for the experiments under relaxing conditions at different ionic strengths, according to Head and co-workers (1995), at an ionic strength below 50 mM the "blocked" state is lost, and the system is no more calcium sensitive. Hence at least 80% of actin should be in the "closed" conformation, as in the presence of calcium (McKillop and Geeves, 1993). Consequently, one would expect some 80% of the cross-bridges that are weakly attached at 30 mM ionic strength to bind to actin in a different conformation compared to their conformation at an ionic strength above 50 mM. Because at 30 mM ionic strength and low temperature at least 60% of the cross-bridges are thought to be weakly attached to actin (Brenner et al., 1986), some 50% of all cross-bridges should undergo structural changes if such changes occur. At high temperature, fiber stiffness is reduced to about two-thirds of its value at 5°C (Kraft et al., 1995a), which means that the number of weakly attached cross-bridges is reduced at most to 40%. Yet more than 30% of all cross-bridges would still be expected to bind to actin in a different conformation as ionic strength is reduced, if changes in the thin filament and, associated with such changes, differences in the mode of weak cross-bridge attachment really occur.

As for changes as calcium is raised in the presence of MgATPγS, mechanical experiments indicate that in this case a large fraction of attached cross-bridges experience the change; otherwise we would not have seen such a prominent shift of the stiffness-speed relation (Kraft et al., 1992). To maximize the fraction of weakly attached cross-bridges, we chose the lowest ionic strength possible for our experiments (10 mM MgATPγS), i.e., 80 mM. Previous stiffness measurements and comparison with biochemical binding studies (Brenner et al., 1986) showed that at 80 mM ionic strength under relaxing conditions, ~40% of the cross-bridges are attached to actin. To get an estimate of the fraction of weakly attached cross-bridges at 10 mM MgATPγS, we compared the intensity ratio of the equatorial reflections, $I_{1,1}/I_{1,0}$, at 80 mM ionic strength under different conditions: in the presence of 10 mM MgATPγS without calcium ($I_{1,1}/I_{1,0}$ is 45% of rigor at 1°C, 80 mM ionic strength) it is about the same as with 10 mM MgATP ($I_{1,1}/I_{1,0}$ is 52% of rigor) and about the same as with 1 mM MgATP at 80 mM ionic strength ($I_{1,1}/I_{1,0}$ is 49% of rigor). The similarity of the equatorial intensities, as indicated by nearly indentical $I_{1,1}/I_{1,0}$ ratios, therefore allows us to conclude that the fraction of cross-bridges weakly attached to actin in the presence of MgATPγS at low calcium should also be at least ~40% of all cross-bridges. At high calcium concentration, the intensity ratio is somewhat higher (Kraft et al., 1992), which indicates a slightly larger fraction of weakly attached cross-bridges. Thus the quite significant fraction of weakly attached cross-bridges is consistent with the rather large stiffness changes seen in the mechanical experiments and supports the interpretation that the lack of large effects in the 2D x-ray diffraction patterns is the result of only a small change in the mode of weak cross-bridge attachment as calcium is raised.

Type of structural change and its implications for the pre-force-generating state

Our results from the x-ray diffraction and mechanical experiments with MgATPγS seem to indicate that the weakly attached cross-bridges assume a slightly altered conformation at high calcium concentration that is different from a rigor-like structure. It should be noted, however, that this result is definitely tied to employing the proper experimental conditions, i.e., that complete saturation of the cross-bridges with MgATPγS must be guaranteed. Under conditions such as high temperature and low nucleotide concentration (1 mM MgATPγS), particularly at high calcium concentration, previously used, e.g., for mechanical studies (Dantzig et al., 1988), or when large bundles are used instead of single fibers (Goody et al., 1975), one finds very prominent rigor features in the 2D x-ray diffraction patterns due to incomplete saturation with MgATPγS. In control experiments we recorded 2D x-ray diffraction patterns at submaximum concentrations of MgATPγS at high calcium concentration which revealed that if only some 10–20% of the cross-bridges are without nucleotide, rigor features can be detected in the diffraction patterns (Kraft and Brenner, 1997).

It was previously proposed (Holmes, 1995) that upon activation of the thin filament by calcium, weakly attached cross-bridges change from an initial nonstereospecific mode of attachment to a "stereospecific weak" interaction. The stereospecific weak interaction was proposed to be the only weak binding state competent for making the transition into the force-generating states. Stereospecific attachment to actin is defined as attachment of the cross-bridges or at least of a part of the myosin head in a specific orientation relative to the actin filament, like, for example, the cross-bridges attached to actin under rigor conditions. Therefore, if such a transition from nonstereospecific to stereospecific attachment would occur, we would expect a redistribution from a more random or myosin-based cross-bridge arrangement to a configuration in which at least part of the cross-bridges follow the actin helix. In 2D x-ray diffraction patterns this would result in a decrease in intensity of the myosin layer lines and a parallel increase in intensity of actin layer lines (e.g., the layer line at 59 Å), as it can be observed, for example, under rigor conditions. A similar change toward a rigor-like pattern would be expected if cross-bridges weakly attached to the activated (unregulated) actin filament had the ordered rigor-like appearance that was observed in electron microscopy by Pollard and co-workers (1993).

However, comparison of 2D x-ray diffraction patterns of single fibers with MgATPγS at low and high calcium concentrations did not reveal changes that would even qualitatively resemble changes toward rigor-like features. The

missing significant enhancement of actin-based features and the missing shift of the 59-Å actin layer line intensity toward the meridian in our diffraction patterns with MgATPγS at high calcium could mean that either the attachment mode of weakly binding cross-bridges does not change toward a stereospecific arrangement, or that only a very small part right at the actin interface assumes an actin-based arrangement, whereas the more distant parts assume a more random orientation within the filament lattice, e.g., because of the mismatch between actin and myosin periodicities. In the earlier electron microscopy work (Craig et al., 1985; Applegate and Flicker, 1987; Walker et al., 1994, 1995), in which myosin heads were free to arrange without the constraints imposed by the filament lattice, cross-bridges attached to the unregulated actin filament in the presence of MgATP (i.e., mainly accumulated in the weak binding $M \cdot ADP \cdot P_i/AM \cdot ADP \cdot P_i$ states; Bagshaw et al., 1974) were found to assume various angles with no preference for a specific orientation relative to the thin filament. In this respect the unregulated actin filament appears to resemble the native thin filament in the presence of calcium.

Taken together with our previous mechanical experiments, the present study, however, seems to provide evidence that upon addition of calcium, the weakly attached cross-bridges with MgATPγS change their mode of attachment to actin, but apparently without large-scale structural changes in the actomyosin complex. When calcium is raised, i.e., when tropomyosin moves and allows access to interaction sites on actin that are thought to be responsible for strong cross-bridge attachment (Lehman et al., 1995), the cross-bridges with MgATPγS apparently also interact with these additional sites—as evidenced by the much slower rate constant for association and dissociation; yet overall affinity is hardly changed. Attachment and detachment kinetics of the cross-bridges very similar to those observed in the presence of MgATPγS at high calcium concentration were also found during unloaded isotonic shortening, where most of the cross-bridges (>90%) seem to accumulate in the weak binding states (Stehle et al., 1993). The much slower rate constant for dissociation indicates a longer lifetime of the weakly attached states in the presence of calcium, presumably because of interaction with the additionally accessible sites on actin. This would be expected to result in a more actin-based orientation of the weakly attached cross-bridges. However, this is not seen, although a rather large fraction of the cross-bridges interact differently, i.e., they should undergo such a change. This might indicate that when the thin filament is activated, only a very small part of a weakly attached cross-bridge, i.e., of a cross-bridge with MgATP, $MgADP \cdot P_i$, or MgATPγS in the nucleotide-binding pocket, assumes a stereospecific orientation relative to the actin helix. Yet, in the case of strongly bound cross-bridges, which in general are cross-bridges without a γ-phosphate in the nucleotide-binding pocket, a large part of the cross-bridge assumes a stereospecific arrangement when interacting with the high-affinity sites on the actin filament, resulting in significant changes of, e.g., the actin layer line intensities, as seen in rigor or during isometric contraction.

Cross-bridges with analogs for weak binding states such as MgATPγS or MgAMP-PNP (Frisbie et al., 1998) show that the presence of a γ-phosphate (or γ-phosphate equivalent) in the binding pocket might make an essential difference in actin attachment of the myosin heads. With both analogs the myosin heads appear not to have the proper configuration to gain binding affinity despite additional interaction with the high-affinity sites that become accessible at high calcium. This is supported by the fact that upon interaction with the additional sites on actin the MgATPγS (or MgAMP-PNP) binding to S-1 becomes destabilized, as seen from the much lower affinity of cross-bridges for nucleotide when MgATPγS (or AMP-PNP) is titrated in the presence versus absence of calcium. In the presence of MgATP, however, after cleavage to $MgADP \cdot P_i$, cross-bridge binding to the additional high-affinity sites on the activated actin filament is facilitated by the release of the γ-phosphate, which allows the cross-bridge to assume a different structure, resulting in high overall affinity (strongly attached) and stereospecificity, allowing the cross-bridge to generate force.

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