Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 5252–5256, June 1993
Biophysics

Structural changes in the actomyosin cross-bridges associated with force generation

(x-ray diffraction from muscle/preforce-generating states in muscle/weak binding cross-bridge states/strong binding cross-bridge states)

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Communicated by Terrell L. Hill, January 28, 1993 (received for review September 1, 1992)

ABSTRACT **It is generally thought that to generate active force in muscle, myosin heads (cross-bridges) that are attached to actin undergo large-scale conformational changes. However, evidence for conformational changes of the *attached* cross-bridges associated with force generation has been ambiguous. In this study, we took advantage of the recent observation that cross-bridges that are weakly attached to actin in a relaxed muscle are apparently in attached preforce-generating states. The experimental conditions were chosen such that there were large fractions of cross-bridges attached under relaxing and activating conditions, and high-resolution equatorial x-ray diffraction patterns obtained under these conditions were compared. Changes brought about by activation in the two innermost intensities, I_{10} and I_{11}, did not follow the familiar reciprocal changes. Instead, there was almost no change in I_{11}, whereas I_{10} decreased by 34%. Together with the changes found in the higher-order reflections, the results suggest that the structure of the attached force-generating cross-bridges differs from that of the weakly bound, preforce-generating cross-bridges and possibly also differs from that of the cross-bridges in rigor. These observations support the concept that force generation involves a transition between distinct structural states of the actomyosin cross-bridges.**

It is generally accepted that force generation in muscle is a result of the cyclic interaction between myosin heads (cross-bridges) and actin while ATP is hydrolyzed. A widely favored structural model for force generation and filament sliding was proposed over 20 years ago (1–3). In this model, the cross-bridge, after attaching to actin, is thought to go through a large-scale conformational change such as a rotation of the myosin head from an orientation perpendicular to the filament axis (90°) to one tilted ≈45°. Force is assumed to be generated by a transition between structurally distinct attached states and hence the idea of conformational changes in the *attached* myosin heads is central to the cross-bridge theory of contraction. Thus far, however, there is yet no direct evidence for the hypothesized conformational change in the attached myosin heads associated with force generation.

In recent years, greater understanding of cross-bridge action in muscle has been achieved. Experimental evidence suggested that in muscle fibers, as well as in solution, actomyosin interactions in the ATP hydrolysis cycle can be broadly divided into two groups: the weak binding states and the strong binding states (4–8). In relaxed muscle, cross-bridges have ATP and/or ADP + P_i bound to the nucleotide binding site (9) and therefore represent, whether attached or detached from actin, the weak binding states. Strong binding states include the main force-generating states. Recent experimental evidence obtained in skinned rabbit psoas muscle fibers suggests that the state immediately preceding force generation is a state in which the cross-bridge is weakly attached to actin (22, 23). This is consistent with the working hypothesis that force generation results from a structural change in the attached cross-bridge associated with the transition from a weakly bound preforce-generating state to a strongly bound force-generating state (4). Accordingly, structural differences are expected between those weakly attached preforce-generating and those force-generating cross-bridges. Therefore, by comparing the structure of the force generating cross-bridges in Ca^{2+}-activated fibers, and the structure of *attached* cross-bridges in relaxed fibers, one may examine an essential part of the hypothesis of cross-bridge action involved in force generation.

In the present study, equatorial x-ray diffraction patterns of skinned rabbit psoas fibers were obtained under relaxing, Ca^{2+}-activated, and rigor conditions. The major departure of this study from earlier x-ray diffraction studies of activated skeletal muscles (10–13) is that the experiments were performed at reduced ionic strength (μ) of 50 mM and at low temperature (5°C) with skinned rabbit psoas fibers. The rationale for the experimental design is that under all three conditions, including the relaxed system, large fractions of the cross-bridges are attached to actin, predominantly in one type of states—i.e., preforce-generating, force-generating, or rigor states. Diffraction patterns will thus allow direct comparison of the attached cross-bridge structures in these three critical states, with greatly reduced complications in interpretation. Evidence is presented here suggesting that the average conformation of the attached cross-bridges during force generation is indeed different from that of the weakly attached ones. Preliminary results have been reported (14).

METHODS AND MATERIAL

Fiber Preparation. Single, chemically skinned rabbit psoas fibers were prepared according to methods described earlier (15–17). Skinning solution contained (mM) 3 ATP, 3 magnesium acetate, 5 KH_2PO_4, 5 EGTA, and 33 CrP (pH 7.0, μ = 145 mM); relaxing solution contained (mM) 10 imidazole, 1 EGTA, 1 Na_2K_2ATP, 3 $MgCl_2$, 10 CrP, and 1 dithiothreitol (pH 7.0, μ = 50 mM); activating solution contained (mM) 1 CaEGTA, 3 $MgCl_2$, 1 Na_2K_2ATP, 10 imidazole, 10 CrP, 1 dithiothreitol, and creatine kinase at 300 units/ml (Sigma) (pH 7.0); preactivating solution consisted of relaxing solution plus creatine kinase; rigor solution contained (mM) 10 imidazole, 2.5 EGTA, 2.5 EDTA, and 30 potassium propionate (pH 7.0, μ = 50 mM). For comparing equatorial intensities under relaxing and activating conditions with that under rigor condition, 5% (wt/vol) dextran T_{500} (Pharmacia) was added to the rigor solution.

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Ca^{2+} Activation. The skinned fiber was fully activated by Ca^{2+} (pCa = 4.5). Sarcomere length = 2.3–2.4 μm. The sarcomere patterns of the activated fibers were monitored by light microscope and laser diffraction immediately before and after x-ray exposures. To maintain stable striation patterns during Ca^{2+} activation, a technique that cycles the fiber repeatedly between isometric force-producing state and isotonic shortening under very low load was used (15). By using such cycling technique, sarcomere striation patterns can be maintained stable for full Ca^{2+} activation even at low μ. The x-ray diffraction patterns of the activated fibers were recorded only during the steady isometric contraction phase.

Equatorial X-Ray Diffraction. Patterns were recorded from single fibers by using the intense x-ray synchrotron radiation source at Deutsches Elektronen Synchrotron (DESY), Hamburg, Germany. Beamline X33 of the European Molecular Biology Laboratory was used. The camera was modified for low-angle diffraction from a small specimen. The specimen-to-detector distance was 3.9 m. Diffraction patterns were recorded on a multiwire linear detector (18). The exposure time for each pattern was generally 5 s.

Experimental Procedures. Diffraction patterns from the same spot of the single fibers were recorded generally in the following order: (*i*) in preactivating solution, (*ii*) in activating solution, (*iii*) in preactivating solution, and (*iv*) in rigor solution (with or without dextran T_{500}). Reversibility was checked by comparing diffraction patterns in relaxing solutions (μ = 20 mM) at the beginning and the end of the entire run. The patterns were fully reversible.

Data Analysis. The diffraction patterns were first normalized by the readings recorded by an ionization chamber placed immediately after the specimen chamber. The readings were proportional to the intensity of the direct x-ray beam transmitted through the specimen chamber and integrated over the duration of exposure. Subsequently, intensities of individual reflections of each pattern were normalized with respect to those of the relaxed state of the same fiber at the same spot. This normalization procedure ensured that the results were not affected by variations in the x-ray beam intensity, fiber size, and length of x-ray path through the specimen chamber (16), so that one may combine intensity data obtained from different muscle fibers under varying camera conditions. Integrated intensities of the reflections were obtained by curve-fitting using a nonlinear least squares fit algorithm, MLAB (19). Each reflection peak [h, k] was fitted to a Lorentzian function with the amplitude, the width, and the centroid as parameters. The background was fitted to a function of two exponentials (16). The data reported for the relaxed fibers were the result of averaging those obtained before and after activation.

RESULTS

At 5°C and μ = 50 mM, skinned rabbit psoas fibers are able to generate force at ≈150 kN/m^2 and the speed of shortening is ≈0.75 muscle length per second (Fig. 1). These mechanical properties are comparable to those found in an intact frog muscle at physiological conditions, indicating that the contractile apparatus in the skinned fiber preparation is functioning normally. Under all three conditions studied, the single skinned fibers show well-defined reflection peaks [1, 0], [1, 1], [2, 0], [2, 1], and [3, 0] that originate from the hexagonal arrays of the interdigitating filaments (Fig. 2) (number of recorded peaks was limited by the length of the detector). The lattice spacing d_{10} is 379 Å for the fiber under relaxing condition, 375 Å under activating condition, and 394 Å under rigor condition without dextran. To minimize effects arising from differences in the lattice spacing, 5% (wt/vol) of the dextran T_{500} was added to rigor solution such that d_{10} was kept constant within 5 Å under all three conditions.

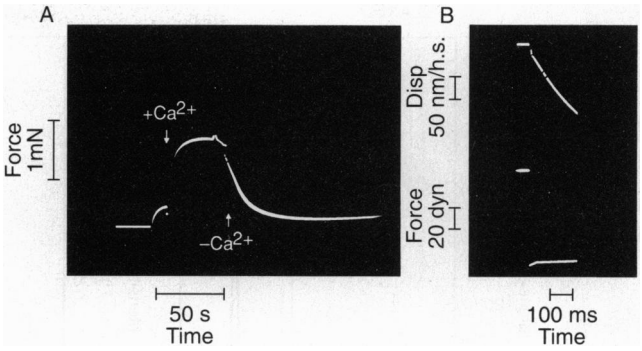

FIG. 1. (*A*) Activation and relaxation of skinned rabbit psoas fibers at μ = 50 mM, 5°C. At the first arrow, preactivating solution was replaced by activating solution. The procedure was reversed at the second arrow. (*B*) Unloaded shortening velocity, μ = 50 mM, 5°C. Force trace shows release to ≈5% maximum force. Shortening velocity corresponds to about 0.75 muscle length per second.

Upon activation, intensity of the [1, 0] reflection, I_{10}, decreases by 34% ± 2% and it decreases a further 35% ± 9% in going into rigor (Table 1). During Ca^{2+} activation I_{20} and I_{21} are weaker than during either relaxation or rigor, whereas I_{30} has a value intermediate of those two conditions. However, the most remarkable feature of the result is that I_{11} is almost constant under all three conditions, contrary to the previously reported reciprocal changes in I_{10} and I_{11} found upon activation of intact frog muscle and skinned rabbit psoas fibers at high μ (10–12, 20, 21), where, in contrast to this study, cross-bridges in the weak binding states under relaxing conditions were mostly detached.

DISCUSSION

The significance of this study is that we compared equatorial diffraction patterns from muscle fibers that, due to our experimental conditions, contain populations of cross-bridges that are known to be mostly attached to actin in single types of states—i.e., either in preforce-generating, in force-generating, or in rigor states. Under the relaxing condition, the weakly attached cross-bridges have been shown to occupy states that apparently are essential intermediates in the pathway to force generation (22, 23). Thus relaxed fibers were used to study the attached preforce-generating states. The low μ and low temperature condition greatly increased the fraction of weakly attached cross-bridges in the relaxed muscle. Based on biochemical *in vitro* measurements, x-ray diffraction data, and fiber stiffness data it has been estimated that at μ = 50 mM and 5°C in a relaxed skinned rabbit psoas muscle, ≈80% of the cross-bridges are attached to actin (16, 24–28). The affinity of force-generating cross-bridges for actin is stronger. Hence nearly all of the cross-bridges are attached during Ca^{2+}-activated contraction. By minimizing the changes in the fraction of attached cross-bridges among the three conditions, ambiguity in interpreting the results—i.e., changes in attachment configuration vs. changes in fraction bound—is greatly reduced. For instance, in skinned rabbit psoas fibers at the higher μ of 170 mM and 5°C, I_{10} decreases by 25%, whereas I_{11} increases by 50% upon full Ca^{2+} activation (21). [Higher orders were not observable due to lattice disorder (16, 29).] Under these conditions, there is an estimated 5- to 10-fold increase (27) in the fraction of cross-bridges attached to actin, which could be the dominating factor in changing the equatorial intensities. Therefore, the present approach allows us to probe more directly for structural changes among the attached states than studies reported previously.

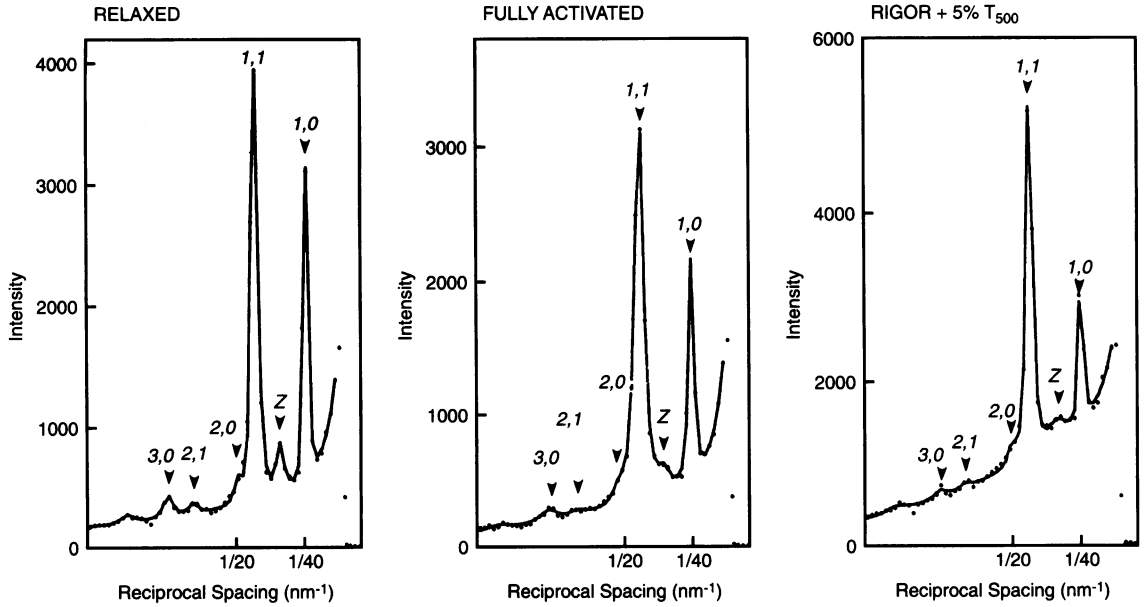

FIG. 2. Equatorial x-ray diffraction patterns from a single chemically skinned rabbit psoas fiber at μ = 50 mM, 5°C. Sarcomere length = 2.3 μm. (*Left*) Relaxing condition. (*Center*) Fully Ca^{2+}-activated condition. (*Right*) Rigor condition with 5% dextran T_{500} added to rigor solution. Exposure time was 5 s for each pattern. Dots, original data; solid lines, curves obtained by nonlinear least-squares fit (16).

Conformational Changes in the Attached Cross-Bridges. One model-independent conclusion is that the differences in the intensities (Fig. 2 and Table 1) recorded under relaxing and activating conditions are attributable to changes in the conformation of the attached cross-bridges. If the fraction of cross-bridges attached to actin were completely unchanged between relaxing and activating conditions, the differences in the intensities would most likely originate from differences in the attachment conformation. However, even if some increase in the attached fraction does occur upon activation, the conclusion would be similar: we have shown earlier (16) that an increase in the fraction of weakly attached cross-bridges in the relaxed muscle caused an increase in I_{11} and a decrease of much smaller magnitude in I_{10}. In the present study, in contrast, activation caused a small increase in I_{11} (3%) but a large decrease in I_{10} (34%) (see Fig. 2 and Table 1). If upon activation only the number of attached cross-bridges had increased without any change in their attachment configuration, the small increase in I_{11} should be accompanied by an even smaller decrease in I_{10}, as it is characteristic for increasing weak attachment in relaxed muscle. In addition, the direction of change in each of the higher-order reflections beyond [1, 1] induced by activation is also different from that induced by attachment of the weak binding type (16). Thus the changes in the intensities cannot simply be accounted for by an increase in the fraction of attached cross-bridges of the same configuration seen in relaxed fibers.

The presence of calcium *per se* is not likely the cause of changes in the diffraction patterns. It was shown that in the presence of adenosine 5'-[γ-thio]triphosphate (ATP[γS]), a slowly hydrolyzable ATP analogue, the skinned fibers exhibited the same mechanical characteristics and equatorial diffraction intensities as the relaxed muscle in the presence of ATP—i.e., the cross-bridges are in the weak binding states. It was further shown that in the presence of ATP[γS], calcium had no significant effect on the intensity ratio I_{11}/I_{10} (30). Therefore, it is unlikely that Ca^{2+} is the cause of changes in intensities.

The apparent width of each individual reflection is somewhat broader during contraction than that found under relaxed condition, indicating that lattice disorder is increased [disorder of the second kind (31)]. It is possible that the observed changes in the equatorial patterns are caused by lattice disorder rather than by conformational changes. If the cross-bridges were to remain in the same conformation during force production as they were in the weak binding states, the characteristics of the diffraction intensities from the weak binding states should be maintained—e.g., I_{10} should remain approximately constant with increasing attachment. However, if we make corrections for lattice disorder by setting I_{10} of the Ca^{2+}-activated state to be the same as that in the relaxed state, then all other reflections after correction would be inconsistent with those observed for increasing weak attachment of cross-bridges (16).

Table 1. Individual intensities and lattice spacing from single skinned rabbit psoas fibers under relaxing, activating, and rigor conditions

Condition	Intensity*					
	I_{10}	I_{11}	I_{20}	I_{21}	I_{30}	d_{10}†
Relaxed	1 ± 0.05	1 ± 0.05	1 ± 0.09	1 ± 0.09	1 ± 0.09	379 ± 4.8
Active	0.66 ± 0.01	1.03 ± 0.04	0.53 ± 0.07	0.57 ± 0.09	0.61 ± 0.03	375 ± 4.8
Rigor + 5% dextran	0.43 ± 0.03	1.09 ± 0.04	0.94 ± 0.13	1.00 ± 0.18	0.44 ± 0.06	380 ± 5.3
Rigor	0.45 ± 0.03	1.25 ± 0.05	0.94 ± 0.07	1.00 ± 0.09	0.66 ± 0.03	394 ± 6.3

μ = 50 mM; temperature = 5°C.

*Individual intensities of the five equatorial reflections [1, 0], [1, 1], [2, 0], [2, 1], and [3, 0]. Intensities were obtained by nonlinear least-squares fit (16). Values are averaged from right and left sides of the diffraction patterns. Ten fibers were used. Errors are standard errors of the mean (n = 10) normalized by the corresponding intensities of the relaxed fibers.

†Lattice spacing of the [1, 0] planes of the hexagonal lattice. Errors are standard deviations.

Our data further suggest that the structure of attached cross-bridges in Ca^{2+}-activated fibers differs from that of fibers in rigor, since the fraction of cross-bridges bound to actin is similar in both cases. This difference is not unexpected since it has long been shown that an activated muscle and muscle in rigor produce distinctly different two-dimensional x-ray diffraction patterns [see, for example, Huxley and Brown (20)].

It could be argued that the observed changes in the equatorial diffraction patterns from relaxed to active to rigor conditions is simply a result of increasing population of cross-bridges in the rigor conformation. Although we cannot exclude completely such a possibility, we believe this is unlikely. If the Ca^{2+}-activated state consists of a mixture of relaxed and rigor conformations only, values of individual intensities of the active muscle should be intermediate between relaxed and rigor, contrary to the data shown in Table 1. Furthermore, recently we reported that radial elasticities of the attached cross-bridges under Ca^{2+}-activated, rigor, and low μ relaxing conditions are distinct from each other (32–34). Such diverse radial elasticities most likely reflect differences in the molecular structures of the attached cross-bridges and/or different modes of attachment to actin (35). Therefore, the idea of a distinct structure of force-generating cross-bridges is consistent with their distinct mechanical properties.

Although the present results suggest structural changes in the attached cross-bridges as force is generated, it is not yet possible to characterize the nature of the transformation unambiguously for two reasons: (*i*) a lack of phase information for the higher-order reflections beyond [1, 1] and (*ii*) artifacts in the reconstructed electron density maps due to limited number of reflections. Relative phase associated with each reflection determines the positions of the scattering units and hence is essential for reconstructing electron density maps. The combination [0°, 0°, 180°, 0°, 0°] appears to be the likely phase set for skinned relaxed muscle at low μ (16), although various phase assignments have been proposed for other muscles and conditions (36–39). The phase set for activated muscle fibers under the same condition, however, is unknown. More importantly, it has been shown that spurious features could be created in the density maps due to limited number (five) of reflections used in reconstruction (40). Even if one uses temperature factors as a way of reducing these cut-off effects, artificial features remain in the reconstructed density maps unless a temperature factor is of such a magnitude that only I_{10} and I_{11} remain significantly above the zero level (L.C.Y., unpublished result). Therefore, without proper modeling, direct interpretation of reconstructed density maps could be misleading.

However, modeling has shown that I_{10} is very sensitive to outward radial movement of cross-bridge mass away from the surface of the thick filament backbone, whereas I_{11} is much less sensitive (40, 54). For example, it was shown that if the center of mass distribution of the cross-bridges moves by 10 Å from the surface of the backbone (from 130 Å to 140 Å from the center of the thick filament), I_{10} decreases by 31%, whereas I_{11} increases by 2%. Thus it appears that once the cross-bridges are weakly attached to actin, in entering the force-generating states there is a slight outward radial shift of mass, probably the part of cross-bridges close to the thick filament backbone, while the part of cross-bridge mass bound to actin is little perturbed. These data together with earlier two-dimensional x-ray studies (48) could well be consistent with an initial weak attachment that occurs at specific sites (22) but the orientation of the major part of the myosin head is variable with respect to axis of the actin filament (i.e., nonstereo-specific binding). This could be accomplished if the weak interaction involves flexible parts on interacting proteins—e.g., the flexible, N terminus of actin (55) and the flexible 20k–50k junction of myosin (I. Rayment, personal communication). Thus, for the weak interaction the mismatch between the periodicities of the thick and the thin filaments may be compensated for without imposing large strain on the attached cross-bridge and the distribution of myosin heads is basically centered around the thick filaments (16). For the higher-affinity, strong binding states, the interaction may involve parts within the myosin and actin that are less mobile (I. Rayment, personal communication; ref. 55), resulting in more restricted angles between myosin head and actin filament axis (i.e., stereo-specific binding). For these interactions the mismatch between actin and myosin periodicity has to be compensated for by deformation at some other area within the crosslink between actin and myosin filaments. An attractive possibility may be the long α-helical tail of the myosin head (I. Rayment, personal communication). Thus, force generation upon the transition from weak to the strong cross-bridge interaction may be visualized as a consequence of transition from a conformation with less well-defined orientation with respect to the actin axis and with little strain to a narrower defined high-affinity conformation with large strain in the cross-bridge.

Some of the observed changes could be induced by attachment of the second head of the myosin molecules to actin, resulting in distortion of both heads. Based on biochemical data (25, 26, 41, 42) and electron microscopic observations (43), cross-bridge binding to actin is likely one-headed under relaxing conditions but two-headed in rigor (44, 45). Activating conditions could result in an intermediate level of attachment of the second head of myosin and it is possible that attachment of the second myosin head contributes to the putative structural change in the attached cross-bridge that drives force generation and filament sliding. However, Table 1 shows that intensities of the five reflections of the activated fibers do not consistently have intermediate values between those of relaxed and rigor fibers, contrary to what would be expected from an increasing attachment of the second head alone. Therefore, we believe that the observed changes in equatorial intensities cannot result exclusively, if at all, from attachment of the second head but also result from structural changes in the cross-bridge shape/orientation.

Relation to Contraction Mechanism in General. The experiments of this study were performed at low μ and low temperature to maximize the fraction of cross-bridges attached in a preforce-generating configuration in relaxed muscle. The results nevertheless have direct relevance to force generation at higher μ and temperature. Preliminary results indicate that even at higher μ and higher temperature, the population of weak cross-bridge attachment to actin, though less favored, is still an essential intermediate in the pathway to force generation (23). Therefore, these findings are highly consistent with the concept that structural changes like those reported here are involved in force generation even under physiological conditions.

In frog and fish muscles, only small fractions of weakly attached cross-bridges have been elicited at low μ and low temperature (46, 47). Thus the question arises whether in these muscles the transition from weakly attached to strongly attached cross-bridge can also play an essential role in force generation. Although the weakly attached states have not yet been demonstrated to be the necessary intermediates to force generation in these muscles, with no evidence to the contrary, it is reasonable to assume that cross-bridges in these muscles (and in general) follow similar steps in the ATP hydrolysis cycles. In all muscle types studied thus far, the actin binding kinetics of the weak binding cross-bridge states in the relaxed fibers are very fast, as indicated by stiffness measurements (24, 28). Model calculations have shown (8) that with fast kinetics, even a small population of attached preforce-generating cross-bridges can function as essential

intermediates—i.e., low occupancy does not necessarily rule out an essential role for the weakly bound cross-bridge states. Therefore, a conformational change in transition from the weakly attached states to force-generating states may well play an essential role in all muscle types.

Relation to Previous Work. Earlier x-ray diffraction results from intact muscles and skinned fibers showed reciprocal changes in I_{10} and I_{11} upon activation (10–12, 20, 21). These results provided compelling evidence that mass shifts away from the thick filament region to the thin filament region and hence are consistent with the idea of an increase in cross-bridge attachment. However, at the time, attached preforce-generating states were not yet identified and whether the weakly attached states were in the force production pathway was not yet determined. Furthermore, for the experimental conditions that were used—e.g., intact frog or skinned rabbit psoas muscle fibers at μ = 170 mM—few cross-bridges were attached under relaxing conditions, and the fraction of attached cross-bridges increased greatly upon activation. Hence, comparisons were generally made between mostly *detached* states and force-generating attached states. Therefore, evidence for structural transformation among *attached* states has been ambiguous. Similar uncertainties complicate the interpretation of earlier two-dimensional x-ray diffraction studies [e.g., Huxley and Brown (20)], although Matsuda and Podolsky (48) and Xu *et al.* (46) showed that myosin layer lines from skinned psoas fibers and skinned frog fibers in the presence of ATP at low temperature and low μ were stronger than those in rigor, suggesting different structures between these two attached states. However, effects of activation under the low μ and low temperature conditions have not yet been determined.

It has been observed in frog and turbot fish fin muscle that during the rising phase of tetanus, changes in I_{10} and I_{11} preceded the rise in tension by 10–30 ms (37, 49–51). For the frog muscle, the differences in the time course were attributed to a "weakly attached," non-force-producing cross-bridge state (52), which was proposed to be different from the weakly bound states described in this paper. However, such weakly attached states have not yet been characterized. For the fish muscle, an attached non-force-producing state was also suggested (37) but not yet characterized. It should be pointed out that during the tetanus rise, several processes are likely to occur simultaneously: movement of detached cross-bridges, attachment, and conformational changes. Even if the time course of projected mass surrounding the thin filament is followed (37), it is difficult to ascertain whether changes in mass distribution are actually related to attachment or to conformational changes. Thus, it is not clear from these observations that structural changes occur with force generation.

Time-resolved studies of the meridional 145-Å reflection following quick releases of an activated frog muscle have been reported (50, 53). The authors attributed the initial drop and subsequent recovery in the intensity of 145 Å to rotation of the attached myosin heads. As it is pointed out by the authors, the transient response of 145 Å to quick length release probably reflects reorientation and redistribution of cross-bridges among force-generating states. Our study, however, is focused on possible structural changes associated with the transition from the low-affinity preforce-generating configuration to the high-affinity force-generating configuration of the attached cross-bridges. The two results are therefore complementary to each other.

We thank Dr. Yuichiro Maeda and members of the European Molecular Biology Laboratory (EMBL), Hamburg Outstation, Germany, for their valuable help in obtaining the data at Deutches Eletronen Synchrotron (DESY), Hamburg, Germany. We thank Mr. Daniel X. Gilroy of the National Institutes of Health for technical help in data reduction. The work was partially supported by Deutsche Forschungsgemeinschaft (BR 8491/1-2,3). North Atlantic Treaty Organization Collaborative Research grants (769/85, 900257) are acknowledged.

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