The Cross-Bridge Mechanism Studied by Flash Photolysis of Caged ATP in Skeletal Muscle Fibers

K. HORIUTI

Department of Physiology, Oita Medical University, Hasama, Oita, 879–55 Japan

Summary: Flash photolysis of caged ATP was first employed in muscle cell physiology about 15 years ago. This technique enables examination of the millisecond dynamics of synchronized cross-bridge ATPases in muscle fibers, and consequently, many photolysis studies have been performed in attempt to elucidate the cross-bridge mechanism. The results of early studies with caged ATP seemed to support the classical “power stroke” theory. However, much of the recent evidence indicates that the contraction in-

duced by the photolysis of caged ATP differs from that predicted by the classical theory. One elusive problem is the biphasic tension development that occurs on the photorelease of ATP in the ADP-rigor fiber in the presence of Ca^{2+} . Another is the long-lasting burst of P_i release after the release of ATP. Further investigations are required to clarify the chemo-morpho-mechanical coupling involved in the cross-bridge reaction. [Japanese Journal of Physiology, 47, 405–415, 1997]

Key words: muscle contraction, cross-bridge, ATP.

Motility, one of the major functions of living cells, is very evident in the contraction of muscle fibers. Among the various types of muscle cell, that of the vertebrate skeletal muscle is the most highly developed in terms of its motile function. In this muscle cell, the contractile proteins are densely packed into the myofilament array, and can achieve rapid tension development or fast shortening. The principal contractile proteins in muscle are myosin and actin, which form thick and thin myofilaments, respectively (Fig. 1B, M and A); these filaments are disposed in a hexagonal lattice arrangement within the myofibril. One part of the myosin molecule (viz., the head) possesses ATPase activity and projects from the trunk of the thick filament to interact with the actin in the thin filament. A myosin head that is either bound or about to be bound to actin is morphologically termed a “cross-bridge.” The contractile force that causes sliding between the filaments is produced by the cross-bridge and fueled by ATP.

In 1957, Huxley [1] proposed a molecular model for the action of cross-bridges which explained the physiological properties of muscle during steady-state contraction. Later, refinements of the morphological

[2], biochemical [3], and mechanical [4] aspects of this model were published. Since then, it has been generally assumed that the functioning of cross-bridge involves an actomyosin ATPase cycle (Fig. 1A) that is synchronized with a structural cycle of detachment and reattachment (Fig. 1B), and that the contractile force is generated by a large angular change in position (or form) of the attached cross-bridge. As a consequence, many studies have been carried out over the last three decades to try to detect angular changes among the cross-bridges. However, no study has yet been successful in demonstrating that a large angular change occurs during tension development in a muscle fiber [5].

The technique of flash photolysis of caged ATP in skinned muscle fibers, which was first reported by Goldman *et al.* [6] in 1982, is a powerful method for studying the cross-bridge mechanism in muscle fibers [7]. By this method, it is possible to rapidly apply ATP to the cross-bridges within a fiber, and mechanically examine the ATP-induced reaction with a millisecond time resolution. As a muscle fiber contains a large array of cross-bridges, it is relatively easy to acquire biochemical or structural information from the cross-

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Correspondence should be addressed to: K. Horiuti, Department of Physiology, Oita Medical University, Hasama, Oita, 879–55 Japan. Tel: +81–975–86–5641, Fax: +81–975–86–5649, E-mail: khoriuti@oita-med.ac.jp

bridges inside the fiber, especially when their reactions are synchronized. By taking advantage of this technical background, various types of studies (Table 1) have been performed on muscle fibers activated by the flash photolysis of caged ATP in attempts to clarify the mechanism underlying the cross-bridge reaction. In this article, I review the observations made in

these studies, correlate them one with another, and point out the problems that need to be solved to achieve a full understanding of the dynamics of the chemo-morpho-mechanical coupling that takes place in ATP-induced cross-bridge reactions.

Basic Mechanics and Its Biochemical and Structural Correlates, as Deduced Using the Classical Theory

In typical muscle experiments involving the photolysis of caged ATP, the muscle specimen used is a skinned fiber (a muscle fiber whose surface membrane has been removed or dissolved so that chemical substances can easily diffuse into or out of the fiber). As a rule, ATP is washed out of the muscle fiber prior to applying and photolyzing the caged ATP. Thus, before photolysis the muscle fiber is in the rigor state (Fig. 2, left-hand side R). A rigor fiber is stiff and bears passive tension which can be varied by a small change in the fiber length.

Mechanical events after photorelease of ATP

The response of a rigor fiber to ATP is relaxation or contraction, depending on whether Ca^{2+} is, or is not present. In the absence of Ca^{2+} , the photorelease of ATP allows the muscle fiber to enter the relaxed state (Fig. 2, X). Under some conditions, the relaxation is delayed by a transient contraction in the midst of the relaxation; this phenomenon will be discussed later in this review. When the transient contraction is canceled

Fig. 1. Cross-bridge reaction: biochemical and structural cycles assumed in the power stroke theory. In **A**, the letters M and A represent the myosin head and an actin monomer, respectively. Four states in a rapid equilibrium are lumped in { }; these are referred to as “detached” states. In **B**, the thick and thin filaments are labeled M and A, respectively, a, the cross-bridge in rigor; b₁, in a detached state; b₂, in a weakly attached state; c, in the state allowing active tension generation.

Table 1. Studies involving flash photolysis of caged ATP in skeletal muscle fibers.

Category	Reference
Kinds of information or events examined	
Tension and stiffness	[6, 8, 9, 11–13]
[³ H]ATP cleavage ([³ H]ADP assay)	[28, 29]
P _i release (P _i -bound MDCC-PBP)	[51]
Electron-microscopic image	[24, 38]
X-ray diffraction pattern	[10, 21–23]
Orientation of fluorescent probe	
Attached to SH ₁ of myosin HC	[36, 37]
Attached to a myosin LC	[5]
Birefringence of the fiber	[39]
Cy3-EDA-ATP fluorescence	[34]
Modifications examined for their effects	
Presence of P _i or V _i	[9, 11, 13, 15, 33]
Presence of ADP	[9, 10, 22, 31, 45, 52]
Intermediate [Ca ²⁺]	[11, 14]
BDM (2,3-butanedione monoxime)	[11]
Different species or fiber types	[10, 16]
Photolysis of caged ATP[γS]	[42]
Variation of ionic strength	[53, 54]
Shortening of muscle fibers	[34, 55, 56]

Fig. 2. Contraction or relaxation initiated by flash photolysis of caged ATP in a skinned muscle fiber in rigor. Records of tension (upper) and stiffness (lower) showing the effects of photorelease of 1.2 mM ATP (from 4.8 mM caged ATP) in a rat psoas fiber at 20°C, in the presence (solid line) or absence (dotted line) of Ca^{2+} . Each photolysis solution contained $10 \mu\text{g} \cdot \text{ml}^{-1}$ type B apyrase. To avoid a delayed relaxation, 8 mM P_i was added to the Ca^{2+} -free solution. K. Horiuti, unpublished results; the methods were the same as in Horiuti *et al.* [10].

out (by the “tension difference” method [8]), the rate of relaxation is approximately proportional to the concentration of the photoreleased ATP. The rate of decrease in stiffness shows similar [ATP]-dependence [9–11]. This ATP-dependence strongly suggests that the time course of ATP-induced relaxation reflects the time course of ATP binding to rigor cross-bridges.

In contrast, when ATP release is performed in the presence of Ca^{2+} , the muscle fiber enters a state of active contraction (Fig. 2, C). An activated fiber is different from a fiber in rigor state: after a stepwise length change, it can recover tension with a characteristic time course [4] (details will be explained later in this review). An interesting point is that ATP-induced contraction is preceded by a brief relaxation (Fig. 2, P) which is similar to the early part of the relaxation seen in the absence of Ca^{2+} [9, 10, 12–14]. The rate of the initial relaxation depends on [ATP] (as in the absence of Ca^{2+}), whereas the rate of the secondary tension development is independent of [ATP] [12]. Since the addition of P_i both suppresses the steady-state magnitude of the contraction and increases the rate constant for its development, the time course of tension development is assumed to reflect the time course of P_i release from the cross-bridges [10, 13, 15].

When mammalian slow- and fast-type twitch fibers are compared, the rate of tension development is found to be slower in the slow-twitch fiber, but the rate of the preceding relaxation is similar in the two fiber types [10]. According to one report about amphibian muscle [16], the tonic fiber relaxes with ATP

at a rate an order of magnitude slower than the twitch fiber. However, this result probably occurred because the pre-photolysis fibers were contaminated with a significant amount of ADP, which inhibits the cross-bridge binding of ATP, and because slower cross-bridges have a higher affinity for ADP [10].

In photolysis trials with Ca^{2+} , photoreleased ATP was consumed by the cross-bridge ATPase and eventually depleted, leaving hydrolysis products ($\text{ADP} + \text{P}_i$) within the fiber. Thus, sooner or later after active contraction, the fiber re-enters the rigor state (Fig. 2, right-hand side R). The photolysis-activated fiber enters the rigor state earlier when less ATP is available to the fiber, and the half-time to rigor state increases linearly with increases in the amount of photoreleased ATP [11].

An interpretation of the basic result

On the basis of (i) the biochemical cycle (Fig. 1 A) proposed for purified actomyosin ATPase in solution, and (ii) the structural information obtained from muscles in equilibrium, the above ATP-induced mechanical reaction has been classically interpreted as follows [17–19].

In rigor fiber in the absence of ATP, the myosin heads are tightly bound to actin, which is polymerized in the thin filament, and form rigor cross-bridges, AM. A strong actin-based periodicity of the cross-bridges can be seen in both X-ray diffraction [10, 20–23] and electron-microscopic (EM) images [24] obtained from rigor muscle; the periodicity is indicative of the tightly bound cross-bridges within the fiber. The electron paramagnetic resonance (EPR) spectra [25] and fluorescence polarization data [26] obtained from probes attached to the myosin head indicate that the myosin heads in the rigor muscle are ordered and that their motion is restricted. In EM images [24], the rigor cross-bridge seems to be in the position it would be expected to occupy just after a “power stroke” [2–4]; this is drawn schematically as in Fig. 1B.

In a muscle relaxed with ATP in the absence of Ca^{2+} , the myosin heads are mostly dissociated from actin, although they may rapidly or weakly interact with each other when the ionic strength is unphysiologically low [27]. Ferenczi *et al.* [28, 29] photolyzed a radioactive caged ATP in rigor fibers, and then followed the time course of ATP hydrolysis by rapidly freezing the fibers for measurement of the radioactive ADP that had been formed within them. ADP appeared rapidly during the initial relaxation that occurred immediately after the release of ATP, and then the rate of hydrolysis became so slow it was negligible in the relaxed muscle. The size of the initial “burst” of

ADP was about the same as the amount of cross-bridges within the muscle fiber. The burst of ADP probably reflects the rapid hydrolysis of ATP in the cross-bridges before an equilibrium is reached. Thus, at any given time, a relaxed cross-bridge is in one of the states in a rapid equilibrium that are lumped in { } in Fig. 1A; among these the $[A+M \cdot ADP \cdot P_i]$ state is the most heavily populated.

The EPR spectra [25] and fluorescence polarization data [26] indicate that, in relaxed fiber, the cross-bridges are disordered and moving rapidly. The X-ray equatorial reflections [10, 20] indicate that relaxed cross-bridges are retracted from the thin filament toward the backbone of the thick filament. Birefringence data from muscle fibers [30] indicate that a relaxed cross-bridge is more nearly parallel to the fiber axis than is a rigor cross-bridge. On the basis of all these observations, a detached cross-bridge should be drawn schematically as b_1 of Fig. 1B. A detached cross-bridge may interact briefly with actin during its rapid motion, as b_2 , but this interaction generates little force.

The photoliberation of ATP within a rigor muscle fiber in the presence of Ca^{2+} induces an initial brief relaxation and then an active contraction, as described above. This two-step response has been explained as follows [12]. An AM cross-bridge binds ATP to enter the "detached" states indicated in Fig. 1B: $\{AM \cdot ATP, AM \cdot ADP \cdot P_i, M \cdot ATP, \text{ and } M \cdot ADP \cdot P_i\}$. It then proceeds to the AM^*ADP state, thus releasing P_i . The cross-bridge in the latter state is the active cross-bridge that bears tension due to its ability to perform the power stroke. The weakly attached cross-bridge (b_2) is thought to change into a strongly bound one (c) during this process of activation [24].

The above two-step reaction is faster than the overall rate of ATPase turnover (approximately 5 s^{-1} under the experimental conditions indicated in Fig. 2 [10]). Therefore, the rate-limiting step in the overall cross-bridge ATPase cycle must occur after P_i -release. It is likely that an active cross-bridge (AM^*ADP) slowly isomerizes into an ADP-rigor cross-bridge ($AM \cdot ADP$) after the power stroke [31]. The activated fiber binds P_i (or its analog, V_i) and becomes less active, but the ADP-rigor fiber scarcely binds either of them. This strongly suggests that the AM^*ADP -to- $AM \cdot ADP$ isomerization is not readily reversible [32, 33].

The release of ADP from the ADP-rigor complex is relatively fast [9, 10, 31]. Thus, while ATP is available cross-bridges undergo a cyclic reaction in which the AM^*ADP must be the most heavily populated state, unless $[P_i]$ becomes sufficiently high to pull an activated cross-bridge back into a detached or weakly-

binding state. Chaen *et al.* [34] studied the time course of ADP release from activated cross-bridges by photolytically applying ATP to a myofibril that had already been activated with a fluorescent analog of ATP (Cy3-EDA-ATP). As the specimen was quite thin, the released Cy3-EDA-ADP rapidly diffused into the bulk solution and made little contribution to the fluorescence of the specimen itself. On photoliberation of ATP, the fluorescence intensity decreased, and the speed of change was doubled when the myofibril was allowed to shorten. Presumably, the slow isomerization of AM^*ADP into $AM \cdot ADP$ is accelerated by a decrease in load. This would be the physiological basis for the load-dependent activity of cross-bridge ATPase ("Fenn effect" [1]).

The Ca^{2+} ion regulates cross-bridge reactions through its effect on the regulatory proteins (troponin and tropomyosin [35]) in the thin filament. This Ca^{2+} -mediated regulation may work by changing the equilibrium between the dissociated and weakly-associated states. In the presence of Ca^{2+} , this equilibrium may be shifted from dissociated ($M \cdot ATP$ or $M \cdot ADP \cdot P_i$) to weakly-associated state ($AM \cdot ATP$ or $AM \cdot ADP \cdot P_i$), resulting in an increased mass flow from lumped "detached" states to the activated state (AM^*ADP). However, the Ca^{2+} -dependence of weak binding is thought to be slight [27]. For this reason, the essential Ca^{2+} -regulated step is assumed to be the P_i -releasing transition from the detached states to the activated state.

Observations not Readily Compatible with the Classical Idea

The results of the photolysis experiments described above do not conflict with the power stroke hypothesis; that is, cross-bridges undergo a series of conformational changes (Fig. 1B) coupled with the biochemical ATPase cycle (Fig. 1A). Only such photolysis experiments can provide values for the kinetic parameters of an *in situ* cross-bridge; these may be different from those obtained using solubilized actomyosin [18]. However, as described below, many recent results from photolysis experiments are not readily compatible with the power stroke hypothesis.

Attempts to detect angle changes among cross-bridges, and the implication of the results

According to the power stroke hypothesis, cross-bridges change angular conformation while generating a contractile force. Several studies have been performed to try to detect this angular change during the contraction induced by the photolysis of caged ATP in

muscle fibers. However, none of the studies have been successful in detecting the angular change during tension development.

Allen *et al.* [5] fluorescently labeled (with acetamido-tetraethyl-rhodamine; ATR) a site in the neck region of the myosin head (regulatory light chain; RLC) in skinned fibers, and then examined the changes in fluorescence that occurred during a caged ATP experiment. They continuously monitored a pair of fluorescence polarization signals to estimate both the amount and orientation of the ordered probes. Fluorescence in the rigor state was highly polarized, reflecting the ordered orientation of the rigor cross-bridges. When ATP was photoreleased, the polarization immediately decreased, probably due to increasing disorder in the cross-bridge orientation. However, during the later phase of the contraction, the polarization changed little. The change in the angle of the probe axis during tension development was estimated to be only about 2° or less.

In fact, the ATP-induced order-to-disorder transition among the cross-bridges was more spectacular than the angular change during force generation in the RLC-labeled fiber [5]. According to one estimation, the fraction of cross-bridges that are disordered is $\approx 45\%$ in the rigor state, $\approx 75\%$ after the release of ATP in the presence of Ca^{2+} , and $\approx 80\%$ in fully relaxed fiber in the absence Ca^{2+} . The order-to-disorder transition starts earlier than a sign of detachment detected mechanically (by the "tension difference" method), suggesting a structural change before detachment. Little change is observed when ADP (instead of ATP) is photoreleased, although ADP has a clear effect in reducing the rigor tension.

Tanner *et al.* [36] examined the ADP-induced structural change by ATR-labeling another site (SH_1 of the myosin heavy chain) in a cross-bridge and monitoring the absorption anisotropy (or linear dichroism; difference in absorption between axially and perpendicularly polarized light). When the axis of the ordered fluorescent probes is more nearly parallel or perpendicular to the fiber axis, the anisotropy is more positive or negative, respectively. However, from this single parameter, one cannot deduce the angle of the ordered probes independently from the fraction of the disordered probes.

The anisotropy of the SH_1 -attached probes quickly reversed the sign (from negative to positive) on the photorelease of either ADP or ATP. The ADP-induced change was similar in size to that induced by ATP, but the former was faster than the latter by a factor of 2 or more when the nucleotide concentrations were matched. On the basis of these results, this anisotropy

is thought to signal a local structural change due to nucleotide binding that is not related to the hypothesized global change involved in active force generation.

Berger *et al.* [37] adopted a technique for monitoring fluorescence polarization [5] and used it in experiments in which ATR was attached to SH_1 [36]. Their study showed that, when either ATP or ADP is photoreleased, the probe axes are disordered with a re-orientation of the principal axis toward the fiber axis. The ATP-induced change occurs far earlier than tension development, and seems to be slightly earlier than the mechanical detachment that occurs in the initial phase. The authors [37] concluded that a structural change at SH_1 may be involved in communication between the nucleotide- and actin-binding sites of a cross-bridge.

Using a rapid freezing technique, Hirose *et al.* [24, 38] performed a time-resolving EM study on muscle fibers activated by the flash photolysis of caged ATP. In their pictures of longitudinal sections, the rigor cross-bridge appears as a tilted triangle. During the relaxation phase immediately after ATP release, the rigor structure was replaced by a thin structure stretching between the thin and thick filaments at variable angles. These variously angled cross-bridges are actually quite similar to those seen in a fully activated fiber.

The structural change during the phase of tension development appeared to be quite small. According to an image analysis of the cross-sectional EM pictures [38], the distribution of the attached cross-bridges among a wide variety of structural classes changed during tension development. This is consistent with the results of X-ray diffraction studies, which will be described later.

A muscle fiber is naturally birefringent. As mentioned above, birefringence is lower in the rigor state than the relaxed state, probably because the long axis of the rigor cross-bridge is more nearly parallel to the fiber axis than that of the relaxed cross-bridge. On the other hand, the difference in birefringence between rigor state and contraction is small. Peckham *et al.* [39] examined the change that occurred in fiber birefringence on the photorelease of ATP in the absence of Ca^{2+} . During the initial relaxation (immediately after the release of ATP), fiber stiffness was greatly decreased but the simultaneous increase in birefringence was disproportionately small. This suggests that a detached cross-bridge is not as relaxed as a steady-state cross-bridge immediately after photolysis.

The EPR spectroscopic method involving caged ATP photolysis could potentially be useful for study-

ing changes in the angle distribution of probes attached to cross-bridges [25]. However, the time resolution of this method is not yet sufficient to enable distinguishing of the two phases (initial relaxation and subsequent contraction) that occur after the release of ATP [40].

Taking all the above findings together, it can be said that (i) the hypothetical large angular change in cross-bridges has not yet been detected during tension development. Instead, it was found that (ii) the non-force-generating, detached cross-bridges observed immediately after the binding of ATP are quite similar to the cross-bridges observed in fully activated muscle fiber. In both the "pre-tension muscle" (muscle after ATP binding and before tension generation) and muscle bearing full active tension, the cross-bridges show a wide variety of orientations and shapes. It should be noted that the data from EPR and X-ray studies on steady-state muscles indicate that only 20–30% of the cross-bridges are tightly bound to the thin filament during contraction [25, 41].

There is another implication of the results of the above studies. The detached cross-bridges seen during photolysis-induced contraction are different from cross-bridges in the relaxing muscle in equilibrium. Therefore, (iii) it is highly probable that the points of action of the regulatory Ca^{2+} reside within the lumped detached states; the weakly associated cross-bridges being shifted to a completely dissociated state by the inhibitory action of the regulatory proteins in the absence of Ca^{2+} .

This idea about the influence of Ca^{2+} on the weak association of detached cross-bridges is supported by the results of a study by Dantzig *et al.* [42] in which caged-ATP[γS] (a photolabile derivative of a non-hydrolyzable ATP analogue) was used. The release of ATP [γS] allows full relaxation of tension in the rigor fiber even in the presence of Ca^{2+} . However, this zero-tension fiber shows significant stiffness in the presence of Ca^{2+} but not in the absence of Ca^{2+} . According to Kraft *et al.* [43], Ca^{2+} has little effect on the actin-affinity of ATP[γS]-bound cross-bridges, only slowing down the on and off rates.

Horiuti *et al.* [13] followed the time course of development of 90° out-of-phase stiffness at a high frequency (200–1,000 Hz) during a photolysis-induced contraction at low temperature (9–10°C). This stiffness is called "quadrature stiffness," and is specific to active contraction, not being observed in rigor or relaxed muscle [12]. The increase in quadrature stiffness occurs more rapidly than the increase in tension or in-phase stiffness. This would be a line of evidence supporting the idea that the detached cross-bridges in the

Fig. 3. Intensity of the X-ray reflections from muscle fiber bundles during a photolysis-induced contraction in the presence of Ca^{2+} . Tension, isometric tension; 11/10, the intensity ratio of the [1,1] to [1,0] equatorial reflections; act1L, the intensity of the 1st actin layer-line (at 36 nm); 144mer, the intensity of the 14.4-nm myosin meridional reflection. Each X-ray signal is normalized with respect to its pre-photolysis value (solid circle), but tension is normalized with respect to its value during the steady-state plateau. The dotted lines indicate the signal levels from the relaxed muscle. ATP (≈ 1.4 mM) was released at $\approx 18^\circ\text{C}$ in a bundle of rat soleus muscle fiber. Type B apyrase ($2\ \mu\text{g} \cdot \text{ml}^{-1}$) was added to the photolysis solution to minimize the contaminant ADP (and ATP) in the caged ATP (5.9 mM). The data were obtained by summing 12 sets of diffraction patterns from different fiber bundles from five rats. The experiments were performed at BL15A, Photon Factory (KEK, Tsukuba) in November 1995 (PFAC approval No. 95G103) (K. Horiuti, N. Yagi, and S. Takemori, unpublished data).

presence of Ca^{2+} are different from relaxed steady-state cross-bridges in the absence of Ca^{2+} .

Structural changes detected by the X-ray diffraction technique

The X-ray diffraction technique is a non-invasive way of acquiring information about molecular structures within a muscle fiber. The equatorial [1,1] and [1,0] reflections arise from a hexagonal lattice of the thin and thick filaments. The intensity of the [1,1] reflection or the intensity ratio of the [1,1] to [1,0] reflections is often used as an index of the fraction of the cross-bridges in the vicinity of the thin filaments. The actin layer-lines arise from the actin helix in the thin filament, and their intensity may be an index of the fraction of the cross-bridges that are stereo-specifically bound to actin, and which are probably in the strong binding state. The 14.4-nm meridional reflection arises from the periodic projection of cross-bridges from the backbone of the thick filament, and

its intensity decreases when the cross-bridges are either tilted or moving rapidly. By using synchrotron radiation as the X-ray source, time resolution becomes good enough to follow the sequential events during photolysis-induced contraction [10, 20, 22]. Several important observations have been made in X-ray diffraction experiments with caged-ATP (Fig. 3).

Immediately after the photorelease of ATP, the intensity of the [1,1] reflection decreases and that of the [1,0] reflection increases [10, 20, 22]; thus, the ratio of the two intensities is greatly lowered. This equatorial change is presumably because of retraction of detached cross-bridges. This cross-bridge detachment is also evidenced by a decrease in the intensity of the actin layer-lines [10, 20]. An important observation is that both the equatorial reflections and actin layer-lines change little in intensity during the secondary phase of tension development [10, 20]. If a considerable fraction of the detached cross-bridges had re-entered the strongly bound states required for tension generation, the intensity of the actin layer-lines would have increased during tension development. In fact, the only intensity change detected during tension development was that in the 14.4-nm meridional reflection from the cross-bridges [20, 22]. Intensity changed little or decreased slightly during the initial phase of ATP-binding, then increased in the subsequent phase of tension development.

The above observations from diffraction experiments indicate the following points (Fig. 4). (i) The distribution of cross-bridges among the detached, weakly-bound, and strongly-bound states differs only slightly between the pre-tension muscle and the muscle with full active tension. Thus, tension development after detachment is not caused by either detached-to-strong or weak-to-strong transitions among the cross-bridges. Therefore, it is probably attributable to either the power stroke of strongly bound cross-bridges [20] or small changes in weakly bound cross-bridges. (ii) Whichever cross-bridge is actually responsible for developing tension, the structural change seems to be such that it results in an increase in 14.4-nm intensity. As shown in Fig. 4, the strongly bound cross-bridges in a pre-tension muscle may become more nearly perpendicular to the fiber axis when performing a power stroke during tension development.

Transient contraction on photorelease of ATP in the absence of Ca^{2+}

When Goldman *et al.* [6] performed the first caged-ATP experiments, they immediately noticed that the rigor fiber did not simply relax in response to ATP release in the absence of Ca^{2+} , but instead exhibited a

Fig. 4. Schematic representation of the cross-bridges at each stage in an ATP-induced contraction. The double contour represents cross-bridges in rapid motion. Note that the periodicity of the cross-bridge tail attachments to the thick filament does not match that of the binding sites for the cross-bridge head on the thin filament.

transient contraction before final complete relaxation. This contraction was larger when the concentration of photoreleased ATP was lower [6, 8, 44]. It became small when P_i was added to the photolysis solution [9, 15] or if the contaminant ADP was removed (by treatment with apyrase) from the photolysis solution [10, 45]. An example of such a Ca^{2+} -free contraction is shown in Fig. 5.

The Ca^{2+} -free contraction ("delayed relaxation") was originally thought to result simply from the action of rigor cross-bridges on detached cross-bridge(s) through the cooperative mechanism described by Bremel and Weber [46]. According to the original idea proposed by Goldman *et al.* [6, 8], when one of the two heads of a myosin molecule forms a rigor cross-bridge and the other is detached from the thin filaments, the rigor cross-bridge (AM in Fig. 1A) accelerates the P_i -releasing strong binding of the detached partner ($\{ \} \rightarrow \text{AM}^*\text{ADP} + \text{P}_i$) even in the absence of Ca^{2+} . This type of cooperative interaction between cross-bridges is plausible, and could produce the Ca^{2+} -free transient contraction seen on the release of ATP in a rigor fiber [6, 8]. Since the Ca^{2+} -free contraction is quite small when pre-photolysis ADP is minimized (though not completely abolished [10]), Thirlwell *et al.* [45] assumed that it is the ADP-rigor cross-bridge that exerts the cooperative action on the detached partner.

Tension overshoot at intermediate concentrations of Ca^{2+}

When Ca^{2+} is present, but at a low concentration,

Fig. 5. Biphasic contraction induced by photolysis of caged ATP in a rat soleus muscle fiber at 8°C. ATP (1.4 mM) was released from 5.4 mM caged ATP in the presence (A) or absence (B) of Ca^{2+} . The stiffness records (dotted lines) are superimposed on the tension records (solid lines). The horizontal broken lines indicate the base-lines. Although the photolysis solution contained no ADP, the pre-photolysis rigor fiber was incubated in this solution for 1 min only; consequently, at the time of photolysis, a significant amount of bound ADP was left within the fiber [10]. P_i (8 mM) was added to the Ca^{2+} -free photolysis solution. The methods were the same as in Horiuti *et al.* [10], except for the experimental temperature (K. Horiuti, unpublished data).

the photorelease of ATP induces a large transient contraction; tension transiently overshoots before settling at a lower level in the steady state [14]. The Ca^{2+} -free contraction described above seems to form a continuum with the transient contraction seen at intermediate levels of $[\text{Ca}^{2+}]$.

Arguably, the tension overshoot should not be surprising since the detached state immediately after ATP release is not the same as the relaxed state. When Ca^{2+} is present at a low concentration, the transition from detached state to relaxed state may be slower than in the absence of Ca^{2+} ; thus, some of the detached cross-bridges may initially enter the activated state before falling into the relaxed state. Indeed, the following kinetic model reproduces the tension overshoot observed experimentally [14]: "rigor" + ATP $\rightarrow$ "detached," "relaxed" $\leftrightarrow$ "detached" $\leftrightarrow$ "activated." In this scheme, Ca^{2+} would regulate only the step between the detached and relaxed states.

Another explanation for the tension overshoot at low $[\text{Ca}^{2+}]$ is, of course, the cross-bridge cooperativity mentioned above. A contribution by this mechanism is plausible since the tension overshoot becomes larger at lower [ATP] [47], as is also the case with the Ca^{2+} -free transient contraction. Future research is needed to elucidate the extent to which the two mechanisms contribute to each transient contraction or tension overshoot. If the cooperative mechanism is the

only cause of transient overshoot, then this overshoot should not be seen when a very high concentration of ATP is instantaneously released at a low $[\text{Ca}^{2+}]$.

Biphasic Tension Development on Photorelease of ATP in the Presence of ADP

When ADP is added to the photolysis solution, the reaction to the photorelease of ATP is delayed, probably because ADP acts as a competitive inhibitor for the released ATP [9, 31]. Apart from this inhibitory effect, ADP has a kind of activating effect on the reaction; the mechanism underlying the latter effect has not yet been clarified. This interesting effect of ADP is best seen under experimental conditions in which the overall time course of the photolysis-induced contraction is slow [10]. An example would be the experiment shown in Fig. 5, which was performed at 8°C on a slow-twitch fiber from the soleus muscle. The soleus fiber has high ADP-affinity, so the contaminant ADP in the photolysis solution greatly affects the ATP-induced reaction [10].

As shown in Fig. 5A, when ATP is photoreleased in the presence of ADP, the fiber rapidly develops tension immediately after photolysis [9, 10]. This contrasts with the rapid decrease in tension in the absence of ADP (Fig. 2). After this early rise, the tension rises more slowly to a steady-state plateau. There is a short pause in tension development between the early and late phases. The late phase of tension development seems to correspond to the main rising phase observed in the absence of ADP. In fact, whereas early tension development is not affected by Ca^{2+} or P_i , late tension development is enhanced by Ca^{2+} and suppressed by P_i [9] (like tension development seen in the absence of ADP). X-ray diffraction data [10] indicate that most of the ADP that is already bound to cross-bridges before photolysis is replaced by the photoreleased ATP during early tension development in the initial phase. As the magnitude of the early tension does not depend on the rigor tension before photolysis, this is a kind of active tension [9].

The authors of some of the early studies [31, 45] proposed several possible mechanisms for the unexpected, early tension development during the replacement of ADP with ATP, although they were not aware of the biphasic nature of tension development. Their hypotheses have not yet received experimental support; rather, currently available data, such as that described above, seems to argue against them [9]. The clear biphasic nature of tension development seen on the release of ATP in the presence of ADP suggests [9] (i) that the replacement of the ADP on cross-

bridges with ATP induces rapid tension development in the "detached" states, and (ii) that relatively slow, P_i -related transition to the "activated" state induces further tension development.

Takemori *et al.* [48] have shown that while ADP intensifies the 14.4-nm meridional reflection from the rigor muscle, it has little influence on the actin layer-lines or equatorial reflections. This indicates that conformation of the rigor cross-bridge changes following the binding of ADP without detachment from the thin filament [48]. The observed ADP-induced conformational change would be the basis for early tension development by rigor cross-bridges after the photorelease of ATP. However, by the time ATP binds to a cross-bridge, the bound ADP must be released. The rigor state of a cross-bridge immediately after the release of ADP may be different from that in the equilibrium rigor muscle. Alternatively, there may be interaction between the ADP-rigor cross-bridges and the ATP-bound cross-bridges [31, 45].

Possible relation between biphasic tension development during photorelease of ATP and the biphasic tension recovery following a change in muscle length

According to the classical theory of Huxley and Simmons [4], the tension-generating process can be subdivided into two events, viz., cross-bridge attachment and its power stroke. It should be emphasized that the power stroke they proposed was assumed to take place quite rapidly, on a sub-millisecond time scale. When the muscle length is quickly shortened in a stepwise fashion and the tension is lowered, the tension loss is rapidly (but only partially) recovered in an early phase that is due to the power stroke of the cross-bridge. The recovery to a full-tension level occurs later at a slower rate. The slackened cross-bridge must be detached from the actin and reattached to another actin to develop tension. Thus, late tension recovery is assumed to be rate-limited by the rate of reattachment of the detached cross-bridge [1].

The biphasic recovery in tension after a rapid change in length appears similar to the biphasic contraction seen after the release of ATP in the presence of ADP. Theoretically, if an ADP-rigor muscle were to be fully activated instantaneously on photolysis, tension development would be like that seen during tension recovery after a quick release, since the myofilament has significant compliance [49]. Experimentally, the rapid recovery in tension after a quick release is faster when [ATP] is higher and [ADP] is lower [50], as is also the case with the early tension development seen in caged-ATP experiments [9]. It is important

that future studies clarify if (and how) the two biphasic elevations in tension are related to each other. The hypothetical conformational change in cross-bridges during the "power stroke" may turn out to occur in the initial rise in tension in caged-ATP experiments.

P_i Release Monitored by a Fluorescent Phosphate-Binding Protein within the Fiber

Recently He *et al.* [51] reported the time course of P_i release after the photolysis of caged ATP. They caused both a fluorescent P_i -binding protein (MDCC-PBP) and caged ATP to diffuse into a muscle fiber, then immersed the fiber in oil. The P_i release within the fiber was assayed in real-time from the changes in fiber fluorescence, as this was intensified on the binding of P_i to MDCC-PBP. P_i appeared with a delay after photolysis, supporting the idea that P_i had not yet been released from the cross-bridges in the pre-active muscle. The P_i release was rapid during tension development, a finding that is more-or-less consistent with the notion that pre-tension cross-bridges release P_i during transition to the activated state.

In connection with the biphasic tension development discussed above, it should be noted that the time course of the P_i release that occurred in the presence of ADP was similar to that seen in its absence, although the overall time course was somewhat slower in the former case than in the latter [51]. The early tension development in the presence of ADP does not seem to be accompanied by early P_i release.

The most puzzling result reported with MDCC-PBP was that the fast rate of P_i release does not seem to be restricted to the first single turnover of the cross-bridge ATPase. The fast rate lasted for several turnovers. Even when tension had leveled off at the plateau, the release rate was much faster than the steady-state rate [51]. This puzzling feature will doubtlessly be solved in further studies with MDCC-PBP.

Conclusion

The schematic diagram in Fig. 4 is intended to summarize the recent data reviewed here. The detached and retracting cross-bridges in the muscle fiber in an equilibrium relaxing state are drawn with double contours to emphasize their rapid movement. The heads of the strongly-bound, rigor cross-bridges (AM) are tilted. It should be mentioned here that the cross-bridge tails are thought to be variably stretched or compressed in the rigor state, as the periodicities in the thick and thin filaments do not match with each other.

When ATP is photoreleased, tension and stiffness in the rigor fiber decrease rapidly. In the absence of Ca^{2+} , the rigor cross-bridges are detached completely and retract back to the relaxed conformation in the state $\text{AM} \cdot \text{ADP} \cdot \text{P}_i$. On the other hand, immediately after photolysis in the presence of Ca^{2+} , many of the detached cross-bridges are disordered, but they stay in the vicinity of the thin filament without generating tension. Even in this pre-active (or pre-tension) muscle, some of the cross-bridges may be taking up relaxed conformation or strongly-bound conformation. The conformational variety among the cross-bridges may be related to the different periodicities of the two filaments [20].

On the transition of pre-active fiber to a tension-generating state, which is believed to be associated with the release of P_i , there is no great change in the averaged structure of the cross-bridges. In fully activated fiber, the cross-bridges are still disordered, thus retaining the structural variety. When the rigor fiber is activated in the presence of ADP, the fiber shows biphasic tension development; it seems that some of the cross-bridges in the "pre-active" muscle can bear contractile activity. Yet to be determined is which of the various cross-bridges in Fig. 4 are releasing P_i and which are bearing the active tension.

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