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An X-Ray Diffraction Study on the ADP-Induced Conformational Change in Skeletal Muscle Myosin

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Effects of ADP on the conformation of myosin cross-bridges were studied in x-ray diffraction experiments on single skinned fibers of frog skeletal muscle by photorelease of ADP from caged-ADP. The experiments were performed at the third-generation synchrotron radiation facility SPring-8 with a time resolution of 5 ms. The intensity of the third-order meridional reflection from myosin filaments (at 1/14.4 nm^{-1}) increased promptly after the ADP release with a time constant smaller than 5 ms, which was similar to that of tension decline. The results show that ADP binding induces a conformational change of myosin in skeletal muscle fibers.

Key words: ADP, caged-ADP, myosin, synchrotron radiation, X-ray diffraction.

The energy of muscle contraction is provided by hydrolysis of ATP (adenosine triphosphate) by muscle protein myosin. Since ADP (adenosine diphosphate) is a product of ATP hydrolysis, it binds to a myosin molecule in the same cleft as ATP does (*1*). Thus it may be expected that binding of ADP changes the conformation of myosin to one similar to an intermediate state in the ATP hydrolysis cycle.

We found that, when ADP is applied to a single skinned frog muscle fiber in rigor, the intensity of the third-order myosin meridional reflection (at 1/14.4 nm^{-1}) increases (*2*). This finding was later confirmed in a bundle of rabbit glycerinated muscle fibers (*3*). This intensity change is considered to be due to a conformational change of myosin heads (cross-bridges) without dissociation from the thin filaments because (i) the intensity change is reversible (*2*, *3*), and (ii) the decrease in tension, which is observed at the same time as the intensity increase, is also reversible (*4*). On the other hand, studies using electron microscopy and EPR spectroscopy detected an ADP-induced conformational change in smooth muscle myosin S1 but not in skeletal muscle S1 (*5*, *6*).

When ADP is applied to a muscle fiber and penetrates it by diffusion, it is difficult to determine how long it takes for ADP to cause the structural change. Although the myokinase (adenylate kinase) activity in the fiber is inhibited by the use of a potent inhibitor AP_5A (diadenosine pentaphosphate), the possibility remains that a part of the effect is due to a small amount of ATP slowly synthesized from ADP by intrinsic myokinase in the fiber. Thus it is not certain that the effect of ADP application is due to binding of ADP to myosin heads.

In the present study, we used caged-ADP (*7*) to examine the time course of the effect of ADP on the cross-bridge structure. Since caged-ADP, like caged-ATP (*8*), can be photolysed by a strong UV light to liberate ADP in a few milliseconds, it can be used to study the rate of action of ADP. The intensity of the third meridional reflection from the thick filament, which has been most commonly measured in muscle diffraction experiments, was studied. As in the case of a rapid muscle length change (*9*), the movement of the myosin heads is so restricted in the present experiment that measurement of this reflection provides valuable, interpretable information.

MATERIALS AND METHODS

Experiments were performed at a small-angle station of BL45XU (*10*) in the third-generation synchrotron radiation facility SPring-8 (Harima, Hyogo). The x-ray wavelength was 0.10 nm. The flux was about 2×10^{11} photons per second. The beam size was approximately 0.4 mm horizontally and 0.2 mm vertically. The X-ray detector was an x-ray image intensifier with a beryllium window (V5445P, Hamamatsu Photonics, Hamamatsu) coupled with a lens system to a fast CCD camera (Hamamatsu Photonics, C4880-80-14A) (*11*). The area of each frame was 640×56 pixels and the time resolution was 4.96 ms (200 frames per second). A fast-decay phosphor (P43) was used in the exit window of the image intensifier to reduce the persistence, which was negligible at the time resolution of the present experiment. The specimen-to-detector distance was 1.8 m.

A single muscle fiber from the sartorius muscle of a bullfrog (*Rana catesbeiana*) was used as the specimen. It was mounted horizontally in a specimen chamber designed for caged-ATP experiments (*12*). The UV light source was a short-arc Xe flash lamp (200 J, λ = 300–370 nm, half-time 0.3 ms) (*13*).

The fibers (length about 3 mm) were treated with 1% Triton X-100 to dissolve sarcolemma and put into rigor in the presence of 20 mM BDM (2,3-butanedione monoxime)

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(*14*). BDM was used to reduce the rigor tension and obtain an ordered diffraction pattern. Then the fiber was stretched by about 1% to observe the tension response clearly. The sarcomere length was about 2.2 μm before the stretch. The photolysis solution contained 0.8 mM caged-ADP (extra high purity, Dojindo Laboratories, Japan), 10 mM Ca-(methanesulphonate)$_2$, 10 mM EGTA (free Ca^{2+} *ca*. 40 μM), 10 mM Mg-(methanesulphonate)$_2$, 15 μg/ml apyrase (Sigma Chemical, Grade VII), 20 mM Pipes-KOH (adjusted to pH 7.0 at room temperature), 10 mM dithiothreitol. The ionic strength was adjusted to 0.20 with K-methanesulphonate. The fiber was washed thoroughly in the rigor solution without BDM and left in this apyrase-containing solution for 5 min before photolysis to remove residual ADP in the fiber (*15*). It was then lifted into the air, and the x-ray exposure and the UV flash were initiated. About 0.38 mM ADP was released. The rate of nucleotide release (*8*) was about 120 s^{-1}. Tension was measured with a semiconductor gauge (AE801, AME, Norway) and recorded digitally at a sampling rate of 1 kHz. The temperature of the fiber was estimated to be 20°C (the ambient temperature, 27°C) (*16*). The photolysis efficiency was chromatographically determined (*13*). The detector was aligned along the meridian (horizontal in the current setup), and the meridional intensity profile was obtained by integrating intensity within 0.061 nm^{-1} from the meridian. Experiments were performed on 24 fibers. With each fiber, the X-ray diffraction experiment was repeated two or three times. The diffraction patterns from these experiments were added together. The intensity of the third-order meridional reflection from myosin (at 1/14.4 nm^{-1}) was measured by subtracting the background, which was obtained by fitting a second-order polynomial function with the constraint that the background decreases monotonically. Intensity was normalized by an average intensity before the photolysis. The data from 24 fibers were then analyzed statistically. The lateral intensity profile (a profile along the equator) of the third-order meridional reflection was obtained by summing intensity in the region 0.063–0.074 nm^{-1} axially. Similarly, lateral background profiles were obtained in the regions 0.063 and 0.074 nm^{-1} axially. The average of the two background profiles was subtracted from the profile across the meridional reflection. The full-width at half maximum of the meridional peak was obtained from this background-subtracted profile.

A preliminary experiment was performed with a multipole wiggler beamline (BL16A) in Photon Factory (Tsukuba). The specimen-to-detector distance was 1.5 m. The storage ring current was 300–360 mA at 2.5 GeV, and the x-ray wavelength was 0.15 nm. The flux at the specimen was about 5×10^{11} cps. The beam at the specimen was about 3.0 mm horizontally, 0.3 mm vertically. The x-ray diffraction patterns were recorded by an X-ray image intensifier (V5445P, Hamamatsu Photonics, with a

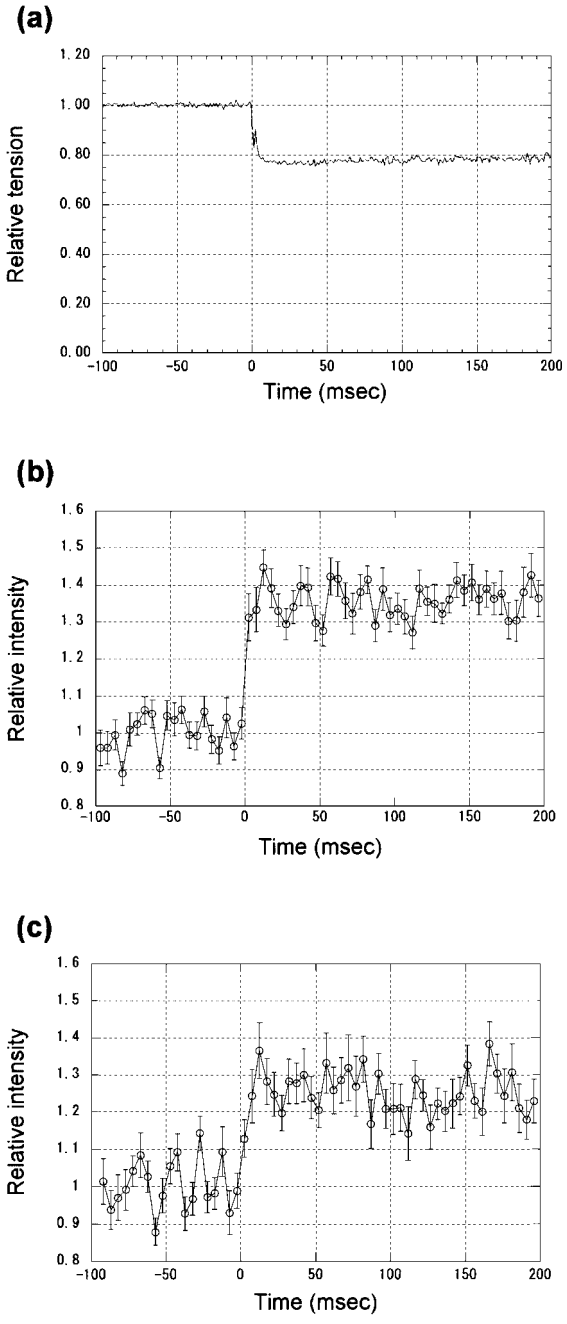

Fig. 1. **(a) A change in tension after photorelease of ADP at time zero.** This is an average of 58 runs on 24 fibers. The tension before the photolysis was 0.76 ± 0.06 mN (mean ± standard error of the mean, n = 24), which was about 30% of the full active tension. The fluctuation immediately after the photolysis is due to noise caused by the UV flash lamp. There is a slow, small increase in tension in 50–200 ms after the flash, whose origin is unknown. It may be due to contaminating caged-ATP in caged-ADP, or ATP formed from ADP by intrinsic myokinase. **(b) Intensity of the third-order myosin meridional reflection after photorelease of ADP.** The error bars indicate the standard error of the mean of 24 sets of data from different fibers. With the data from each fiber, intensity in each frame was normalized by the average before the photorelease. Then the results were analyzed statistically. The mean intensity before the flash was 1,370 photons/frame. **(c) Intensity of the third-order myosin meridional reflection after photorelease of ADP without stretch.** The error bars indicate the standard error of the mean of 14 sets of data from different fibers. The intensity of the meridional reflection was lower because the enhancement due to stretch was absent (*3*). The mean intensity before the flash was 700 photons/frame. The lower intensity was the major reason for the poorer statistics compared with (b).

P20 phosphor in the exit window) coupled through optical lenses to a CCD video camera (XC-77R, Sony, Tokyo). See Yagi *et al*. (*12*) for technical details and a typical diffraction pattern recorded using this detector system. The time resolution was 16.6 ms. The detector had persistence: when the detector had been illuminated by an X-ray beam and the beam was cut off at the beginning of a frame, the first 16.7-ms frame showed about 25% of the intensity before the cut-off, the next frame 12%, the third 9%. This behavior of persistence was not strictly exponential, suggesting that more than one process may be involved. The experimental protocol was the same as the experiment at SPring-8. The specimen was a single glycerinated muscle fiber from rabbit psoas muscle. Data were obtained from six stretched and six unstretched fibers.

Results and Discussion—On an UV flash, the tension dropped to 79.2 ± 1.5% (mean with a standard error of the mean, n = 24) in about 10 ms (Fig. 1a). Although the tension traces recorded in the X-ray experiments suffered from a large artifact due to the flash, a separate experiment in the laboratory under the same conditions showed that the time constant of the tension drop was about 3 ms.

The X-ray intensity increase of the third myosin meridional reflection (Fig. 1b) was also found very fast. In the first 5-ms frame after the photolysis, the intensity was already more than 30% higher than the average level before the photolysis. The time constant, obtained by fitting a single exponential function to the mean values using KaleidaGraph (Synergy Software, USA), was 1.5 (±0.6) ms. The intensity settled to a level 36 ± 2% higher. The increase in the present experiment is smaller than in the previous experiment (*2*), in which about 100% increase was observed. The reason is not clear, but the intensity before the application of ADP may be higher because of the use of BDM. Takezawa *et al.* (*3*) observed an even smaller intensity increase, 23%, in rabbit glycerinated muscle fibers.

No significant change in the spacing of the meridional reflection was found (the ratio of the spacing before and after the photorelease was 1.000 ± 0.001). The peak profile may have changed (Fig. 2), but the precise nature of the change was hard to determine at the spacial resolution of the present study. The intensity of other reflections was too low to be measured reliably. An UV flash in the absence of caged-ADP did not cause any change in the diffraction pattern.The experiment was also done without stretch. The tension was not measurable, because BDM suppressed rigor tension almost completely. The size of the intensity change was smaller than with stretch, 25 ± 3% (n = 14). The intensity change (Fig. 1c) was best fitted with an exponential curve with a time constant of 2.8 (±1.6) ms, which was not significantly different from the value in stretched fibers. Thus, the fast intensity change was not due to the high tension or stress in myosin heads. The larger intensity increase may be related to a conformational change of myosin heads induced by stretch in the rigor state. Takezawa *et al*. (*3*) observed that the intensity of this meridional reflection increased when a rigor muscle was stretched, showing that the conformation of attached heads is sensitive to the stress. In their modeling, binding of ADP and stretch caused a similar conformational change in the tail portion of a myosin head. Thus, it is plausible that stretch affects the conformational change induced by ADP binding.

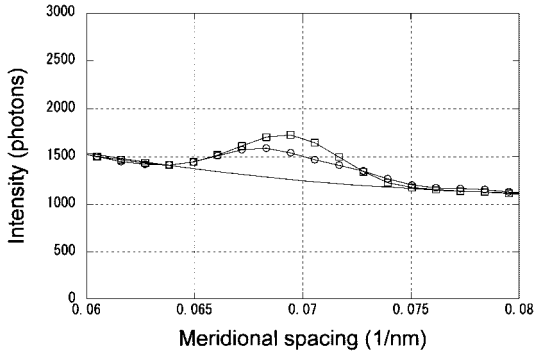

Fig. 2. **The meridional intensity profile from a skinned frog muscle fiber.** The peak is the third-order meridional reflection from the thick filament. This is a typical diffraction pattern obtained by adding images from three experiments on one fiber. A profile before the photorelease of ADP (open circles) and that in 0–5 ms after the photolysis (open squares) are plotted. The background drawn for the latter is also shown.

The lateral width of the meridional reflection was 0.015–0.016 nm^{-1} (full width at half maximum), which did not change after photorelease of ADP either with or without stretch.

A preliminary experiment at Photon Factory was made at a time resolution of 16.7 ms on glycerinated rabbit fibers. The time constant of the intensity change was 100–150 s^{-1} with and without stretch. This is in the region of the time constant of the phosphor persistence in the detector used in the experiment (see "MATERIALS AND METHODS"). Thus, the intensity change seemed to be complete within the time resolution of the detector system. This is consistent with the newer results presented above, which were obtained by using a detector with shorter persistence.

The present results show that the ADP-induced conformational change of the actin–bound myosin heads is a fast process and likely to be caused by binding of ADP itself, not by ATP slowly synthesized from ADP by myokinase or detachment of cross-bridges. The X-ray intensity change is as fast as the tension drop, and faster than the change in fluorescence from a label attached to myosin heads in a rabbit skeletal muscle fiber (*7*) (123 s^{-1} with 50 μM ADP at 18–24°C). The time constant of 1.5 or 2.8 ms found in the present study is in fact too fast, considering that the rate of ADP liberation (*8*) was 120 s^{-1} and that the amount of released ADP was similar to that of myosin heads in the fiber [*ca.* 150 mM (*17*)]. It is possible that ADP binding to some myosin heads, for instance, those which are more tilted, is faster than that to other heads. This would explain the fast time course of the intensity change, because a conformational change of such heads may affect the meridional intensity more effectively than others. However, the large scatter of data in the present experiments prevents us from drawing firm conclusions from the comparison of time courses of the tension and intensity changes.

As for the structural change in myosin heads, Danzig *et al.* (*4*) discussed that the tension decline on addition of ADP to rigor fibers would correspond to approximately

0.2 nm of filament sliding. A possible conformational change of myosin heads upon ADP binding was discussed by Takezawa *et al*. (*3*), who showed that a structural change in the tail portion of the myosin head is consistent with the x-ray results. The present results demonstrate that ADP binding can directly induce these structural changes in skeletal muscle fibers, despite the fact that only very small changes were observed in other studies (*5*, *6*). In those studies, an S1 fragment of myosin, not the entire myosin in the thick filament, was used. Thus the difference is likely to be due to the presence of connection to the thick filament backbone.

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