

# Time-resolved x-ray diffraction study of the troponin-associated reflexions from the frog muscle

Yuichiro Maéda,\* David Popp,<sup>‡</sup> and Alexander A. Stewart\*

\*European Molecular Biology Laboratory at DESY, D-W2000 Hamburg 52; and

<sup>‡</sup>Max Planck Institute for Medical Research, D-W6900 Heidelberg, Germany

**ABSTRACT** The vertebrate skeletal muscle gives rise to a series of x-ray reflexions indexed as orders ( $n$ ) of 77 nm, the even orders being meridional whereas the odd orders being near-meridional. The diffraction intensities associated with these reflexions originate from the axial period of 39 nm attributable to the repeat of troponin-tropomyosin on the thin filament. In the present study, the x-ray intensities of the furthest inner reflexions, A2 ( $n = 2$ ) reflexion at an axial spacing of  $1/39 \text{ nm}^{-1}$  and A4 ( $n = 4$ ) reflexion at  $1/19 \text{ nm}^{-1}$ , of this series were measured with a time resolved manner. Upon activation of the frog striated muscle, the two reflexions underwent biphasic time courses of the intensity changes. With A2 reflexion, a rapid intensity increase by 16%, being completed by the time when tension rises to 5%, was followed by a slow intensity decrease down to 50%, which was associated with the tension rise. In both phases, lateral widths remained unchanged. A4 reflexion also behaves in the same way, although the first phase (the intensity increase) was not clear due to unsatisfactory statistics. We interpret phase one as being caused by conformational change of the troponin-tropomyosin complex upon binding of  $\text{Ca}^{2+}$  to troponin, whereas phase two being due to direct contribution of the mass of the myosin heads bound to the thin filament, although possible contribution of conformational changes of the regulatory proteins to phase two is not excluded. The results indicated that the calcium activation of the thin filament leads the onset of the actomyosin interaction.

## INTRODUCTION

In vertebrate skeletal muscle, the thin (actin) filaments play essential roles both in muscle contraction and the calcium regulation. Muscle contraction is caused by the sliding of the thin filaments relative to the thick (myosin) filaments. The thin filaments, especially proteins troponin and tropomyosin (the regulatory proteins) residing on the thin filaments, are responsible to the calcium regulation. Therefore, knowledge of the thin filament structure and its changes on activation must be of great importance for understanding the mechanisms of muscle contraction and the calcium regulation.

On x-ray diffraction patterns from muscle, some reflexions have been assigned as of the thin filament origin, mainly based on the spacings of the reflexions. Structural repeats in the thin filaments are different from those of the thick filaments (Huxley and Brown, 1967). From some of the thin filament-associated reflexions, time courses of intensity changes have been recorded; 5.9 and 5.1 nm layer-lines (Wakabayashi et al., 1985; Kress et al., 1986; Maéda et al., 1988), the outer part of second actin layer-line (at  $1/19 \text{ nm}^{-1}$ ) (Kress et al., 1986; Maéda et al., 1988), both from frog muscle, and the inner part of first actin layer-line from arthropod muscles (Maéda, 1986; Maéda et al., 1986, 1988; Griffiths et al., 1988).

In the present study, another series of thin filament-associated reflexions were investigated which are indexed as orders of 77 nm, originating from the troponin repeat on the filament.

The intensities of these reflexions are limited on the meridian, indicating that these reflexions are Fourier transforms of the distribution of the mass projected onto

the filament axis. Second order (at an axial spacing of  $1/38 \text{ nm}^{-1}$ ) of this series on the pattern from rabbit glycerinated muscle was intensified when the muscle was treated with anti-TnC antibody (Rome et al., 1973). On patterns from crustacean muscles, 15 reflexions of the same series were observed and the intensities of them are characteristically modulated. This intensity modulation has been interpreted (Wray et al., 1978; Maéda, 1979; Maéda et al., 1979; Namba et al., 1980) in terms of the model for troponin arrangement on the thin filament proposed by Ohtsuki (1974) as a revised version of Ebashi's model (Ebashi et al., 1969). It is worth noting that, in theory, any periodic alteration of the mass associated with the thin filament, whether it is of actin, tropomyosin, troponin or of the cross-bridge origin, would contribute to the reflexion intensities as far as the axial repeat is an order of 77 nm, although the major contribution to the intensity (in the resting state) is likely made by the periodic locations of troponin molecules.

This series of reflexions are different in the following features from other thin filament-associated reflexions. First, these reflexions report changes of the mass distribution in the axial direction, whereas other off-meridional layer-lines are sensitive to structural changes which has helical symmetries. Second, a number of reflexions of this series are observable, facilitating the interpretation of intensity changes. On the pattern from frog, at least three reflexions are observable, whereas from crustacean muscle even more than 10 reflexions of the same series are observed. Third, some of these reflexions, especially second and fourth, are more intense than the 5.9-nm layer line, being intense enough to measure time course with a time resolution of 10–20 ms.

In the present study, we have analyzed the time course of the intensity changes of the first two reflexions of this

Address correspondence to Dr. Maéda.

Dr. Stewart's present address is Rosenstiel Center, Brandeis University, Waltham, MA 02254-9110.

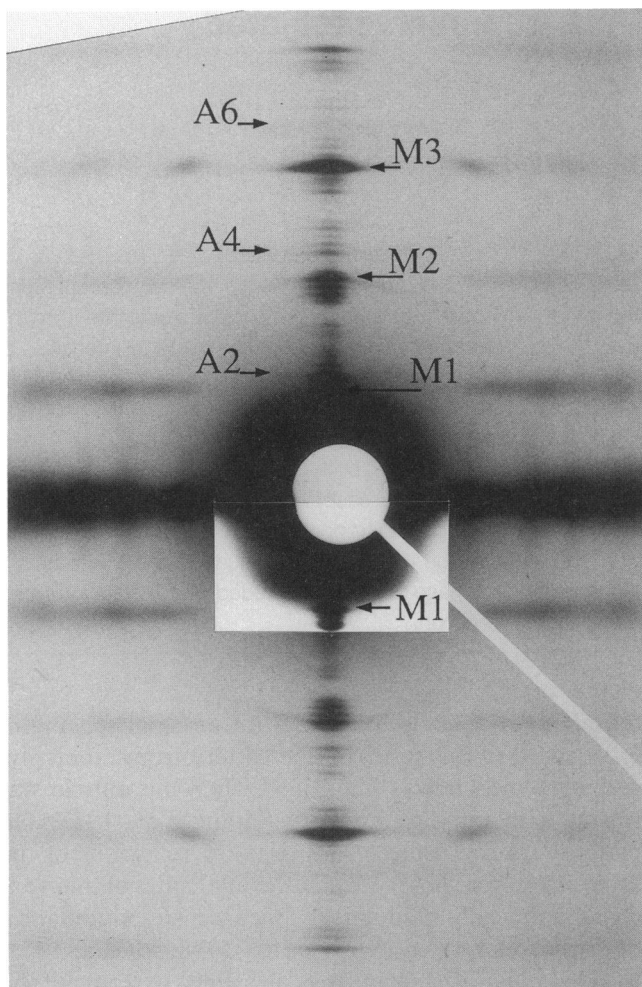


FIGURE 1 An x-ray diffraction pattern from a live frog sartorius muscle in the resting state at 4°C. *M1* ( $1/44.22 \text{ nm}^{-1}$ ), *M2* ( $1/21.56 \text{ nm}^{-1}$ ) and *M3* ( $1/14.34 \text{ nm}^{-1}$ ) are thick filament associated reflexions, the numbers in parenthesis being axial spacings of the reflexions. *A2* ( $1/38.92 \text{ nm}^{-1}$ ), *A4* ( $1/19.24 \text{ nm}^{-1}$ ) and *A6* ( $1/12.8 \text{ nm}^{-1}$ ) are thin filament associated reflexions. The spacings were calculated relative to *M3* reflexion the spacing of which has been calibrated (Haselgrove, 1975). The lower half of the central part was reproduced from the same original in a different contrast to show *M1* reflexion. Beam line EMBL X-33 was used as an intense x-ray source. The x-ray optics were carefully optimized for a fine focus and for the low angle region down to  $1/100 \text{ nm}^{-1}$ . The pattern was recorded on a Kodak DEF-2 no-screen film.

series from live frog muscle and found that the intensities of these reflexions change in two phases upon activation. We interpret that the first increasing phase originates from conformation changes of the Tn-Tm complex induced by the  $\text{Ca}^{2+}$  binding, whereas the second decreasing phase is attributed to the formation of cross-bridges which fill the gap between two successive troponin levels.

## METHODS AND MATERIALS

X-ray diffraction patterns were recorded at station EMBL X 33 situated in Hamburger Synchrotronstrahlungslabor (HASYLAB) of

Deutsches Elektronen-Synchrotron (DESY). The beam line receives x-ray photons from the electron orbit of electron-positron storage ring DORIS. During the experiments, the ring stored only electrons of 50 to 95 mA at 3.656 GeV. The focusing x-ray optics at X-33 was described previously (Kress et al., 1986; Maéda et al., 1986). The wave length of the x-ray beam after monochromator was  $\sim 0.155 \text{ nm}$ . The x-ray optics including slits were carefully optimized for fine focus and for a low angle region down to  $1/100 \text{ nm}^{-1}$ , since it was essential in this study to resolve reflexions which are closely spaced either from each other or from the direct beam. The focus of  $\sim 1.5 \text{ mm}$  vertical and  $4.4 \text{ mm}$  horizontal (both in full width of 10% maximum, which roughly corresponds to  $4 \times \sigma$  of a Gaussian distribution) was made on the detector which was  $\sim 4.5 \text{ m}$  behind specimen. To record the reflexion intensities, a multiwire linear counter (Hendrix et al., 1982) was used which was placed either on the meridian or crossing the meridian. When placed on the meridian, a slit  $10 \text{ mm}$  wide limited the lateral aperture to  $\pm 1/140 \text{ nm}^{-1}$  either side of the meridian, accepting the full lateral width of the reflexions. To record the lateral intensity distribution of individual reflexions, the detector was placed crossing the meridian with a slit  $2.5 \text{ mm}$  wide limiting the axial aperture to  $1/280 \text{ nm}^{-1}$ . In either case, the full length of the sensitive area  $128 \text{ mm}$  long was used. The spatial resolution of the detector is  $1 \text{ mm}$ , which is determined by the spacing of wires. The data acquisition system and the data analysis software were the same as in the previous works (Kress et al., 1986; Maéda et al., 1988).

Sartorius muscles were dissected from frogs *Rana pipiens*, and mounted in the specimen chamber which has been previously described (Maéda et al., 1988). Care was taken not to allow muscle to move on activation. A series of electric pulses of alternating polarities at  $20 \text{ Hz}$  were applied for  $300\text{--}600 \text{ ms}$ , to stimulate muscle to a tetanic contraction under a fixed-end condition. The stimulation was repeated every  $3 \text{ min}$  and x-ray counts were accumulated from 10 to 20 contractions of one muscle. All the data were collected at  $4\text{--}6^\circ\text{C}$ .

Out of 26 muscles used, data from 18 muscles were rejected for one of the following reasons. Under the conditions of the present study, some muscles produced tension either of a low level or with a slow time course. In some muscles the meridional reflexions are weak relative to the central scattering. Muscles were also rejected which showed appreciable movement artefact of muscle in the beam so that not only reflexion intensities but also the background intensity (in the region further outside than  $1/60 \text{ nm}^{-1}$ , see below) undergo substantial changes. No way was found to correct for the movement artefact (see below). 14 accepted data sets from eight muscles were individually analyzed. These were indistinguishable from each other in tension time course, in x-ray diffraction patterns and in time courses of major reflexions intensities. Then these data sets were added up to obtain better statistics.

X-ray patterns of 128 channels were accumulated during each contraction cycle in successive time frames of  $10$  or  $25 \text{ ms}$  duration. These were stored in separate memory positions and summed over contraction cycles. The time courses of reflexion intensities, without the background intensities, were obtained by simply adding up the counts of corresponding channels, then by subtracting the same background intensity from each time frame, being based on an assumption of constant background. The assumption is justified by our observations that the intensities between reflexions, both sides of peaks *M3*, *A4* + *M2*, *A2* + *M1* as best seen in Fig. 1 *A*, remained almost unchanged on activation. This is the case in the region further outside than  $1/60 \text{ nm}^{-1}$ .

The "background" further inside than  $1/60 \text{ nm}^{-1}$ , however, showed enormous decrease in intensity. The decrease is likely caused by decreases in intensities associated with two kinds of reflexions; a series of closely spaced reflexions (Huxley and Brown, 1967) which were not resolved with the present system (the x-ray optics with the detector), and the diffuse off-meridional reflexions which extends to the meridian (Zappe and Maéda, 1985). Due to the enormous intensity decrease in this area, the integrated intensities over the spectrum did not remain constant during activation, making it impossible to correct the patterns for the movement artefact, as described above.

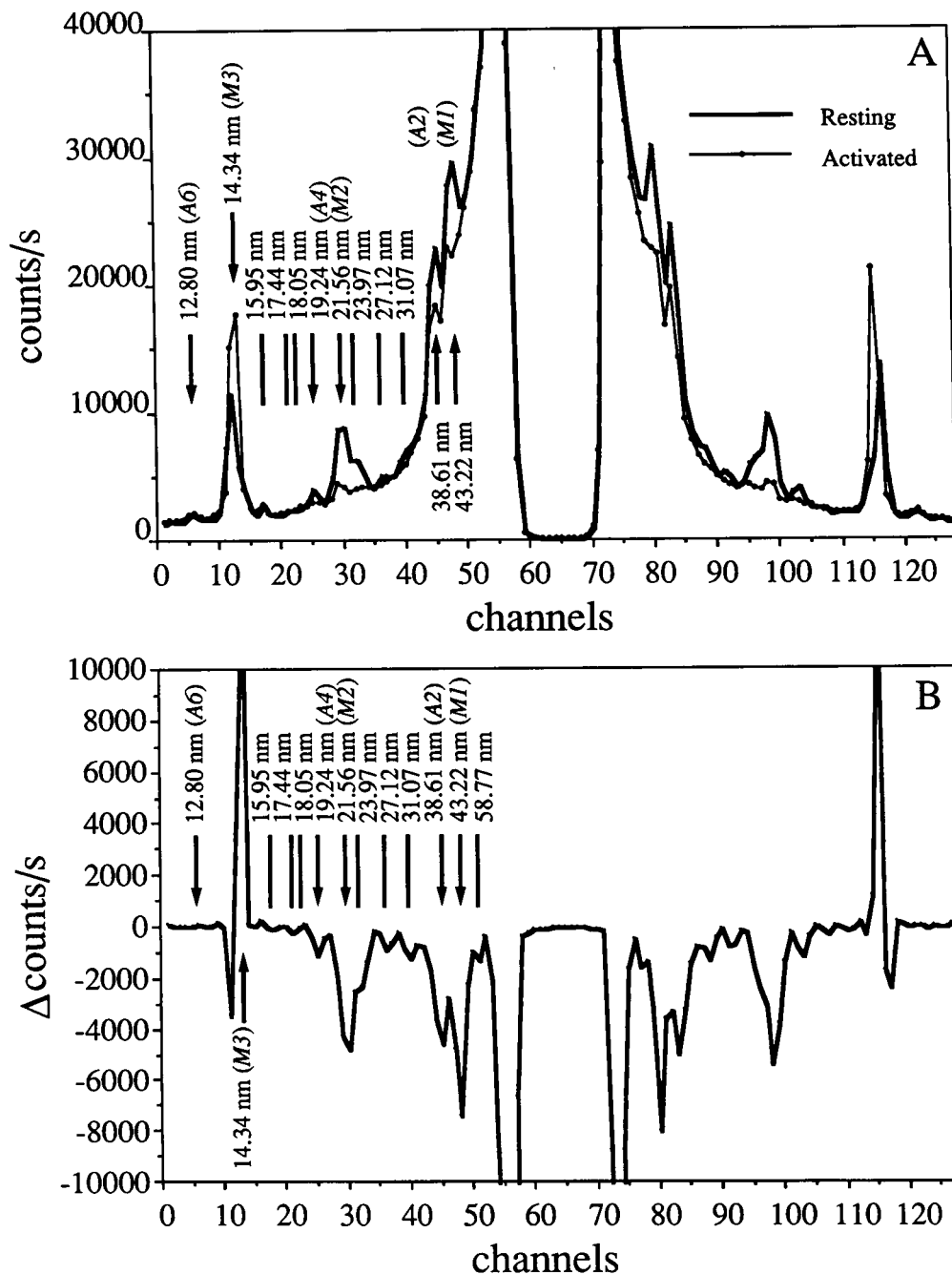


FIGURE 2 (A) Meridional x-ray diffraction spectrum recorded with a linear detector from frog muscles in the resting state (*thick line*) and from the same muscle in tetanus plateau (*thin line*). (B) The difference pattern, (tetanus plateau) minus (resting). Each reflexion is labeled with the spacing in nm at the center of the peak together with the assignment of the reflexion in parenthesis. The spacing labeling *M3* reflexion is that in the resting state. The ordinate, x-ray counts per second. The abscissa, number of channels. In the further right side is the shadow of the beam stop. Patterns were collected from two muscles, total 26 contraction cycles (Z42, MAY89TN).

## RESULTS

### Meridional reflexions on film and with detector

Fig. 1 shows an x-ray diffraction pattern from the frog sartorius muscle at 4°C. The pattern was recorded on a film, otherwise under the same conditions for the time-

resolved experiments using the linear detector. The major reflexions are labeled with *An* or *Mn* where *A* denotes a thin filament associated reflexion, *M* a thick filament associated reflexion and *n* is an integer indicating *n*th order the principal repeats of each filament, 77 nm for the thin filament whereas 43 nm for the thick filament. These reflexions have been assigned (Huxley and Brown, 1967; Haselgrove, 1975), while some of the reflexions

TABLE 1 Intensity changes of major meridional reflexions on activation at 4°C

Reflexions spacing	Assignment	Relative intensity <sup>‡</sup>		Changes <sup>§</sup>	Time to <sup>†</sup>	
		Resting	Tetanus plateau		50% change	90% change
<i>nm</i> *					<i>ms</i>	<i>ms</i>
38.6	A2	1.02	0.53	increase to 1.16 decrease to 0.53	peak at 40–50 88	180
19.2	A4	0.14	0.04	increase to 1.1 decrease to 0.30	peak at 40–50 88	160
43.2	M1	1.11	0.45	decrease to 0.41	40	63
21.5	M2	0.61	0.08	decrease to 0.20	63	110
14.34/14.6	M3	1.00	2.16	increase to 1.48	63	88
Tension				Rise	88	180

\* Spacings are calculated relative to *M3* reflexion in the resting state which has been calibrated as 14.34 nm (Haselgrove, 1975). <sup>†</sup>The integrated x-ray intensities after subtracting the background are measured by planimetry of the patterns presented in Fig. 2. The reflexion intensities are all relative to the *M3* intensity in the resting state, which was  $1.0 \times 10^4$  counts/s on one side of the origin. <sup>‡</sup>The ratio represents the highest or lowest intensity during each phase relative to the intensity in the resting state. <sup>§</sup>Time after the first stimulating pulse. The same data sets as for Fig. 2.

which are weak and unlabeled, like one at  $1/17.1 \text{ nm}^{-1}$  and another at  $1/18.0 \text{ nm}^{-1}$ , had not been previously resolved.

In Fig. 2 *A* are shown a pair of meridional x-ray intensity profiles superimposed, which were recorded with the linear electronic detector; one from the muscle in the resting state and the other from the same muscle during the tetanus plateau. Fig. 2 *B* is a difference spectrum, the pattern in plateau minus that in the resting state. The patterns cover a range of reciprocal spacing from  $1/12$  to  $1/70 \text{ nm}^{-1}$  in which 13 peaks are resolved, each being labeled with spacing at the center of the peak. Closely spaced branches of doublets or triplets shown in Fig. 1 are not resolved here, mainly because of a limited spatial resolution of the detector used. Nevertheless, the major reflexions here indicated by arrows are well resolved from the adjacent reflexions, making it possible to measure the intensities associated with individual reflexions separately. Note that, except for the extreme vicinity to the backstop (see Materials and Methods), the background remained fairly constant on activation, as best seen at a spacing of  $1/60 \text{ nm}^{-1}$  where the difference spectrum is close to zero.

### Intensity changes at 4°C

In Table 1 and Fig. 3, the results are summarized of the time courses of the intensity changes of the major reflexions at 4°C with a time resolution of 25 or 12.5 ms. *M* reflexions and *A* reflexions behave completely differently from each other.

A2 reflexion, which was as intense as *M3* in the resting state, changed its intensity in two phases, first increased and then decreased. In first phase, the intensity increased by 10 to 16% of the reflexion intensity in the resting state. In each separate data set, this increase was significant, since in the resting state, i.e., in 17 time frames preceding the initiation of stimuli, the mean deviation of the counts was  $\sim 1.5\%$  of the mean. This phase was extremely fast; the intensity reached its maximum at 40–

50 ms after the first stimulating pulse. At this moment, tension had not reached to 5% of the maximum, *M2* and *M3* intensities to  $\sim 20\%$ , whereas *M1* had already decreased to a half of the level during the plateau. This fast increase in intensity was followed by the second decreasing phase, which was associated with the tension time course. The over-all shape of the time course of the A4 reflexion intensity was similar to that of A2, although the fast increase in intensity was not substantial above the statistical fluctuation of the resting level (in individual data sets, the increase were  $\sim 10\%$ , whereas the fluctuation of the resting levels was  $\sim 5.5\%$ ).

The time courses of intensity changes of the *M* reflexions were similar to each other, all substantially leading the tension rise, although the directions of the changes were different; *M1* and *M2* decreased while *M3* increased. *M3* showed substantial spacing change too. The observations of *M2* and *M3* had been reported previously (Huxley et al., 1982), while the time course of *M1* was measured for the first time. Time to 50% change of *M1* (decrease) was 40 ms which was somewhat shorter than those of *M2* (decrease) and of *M3* (increase), both being 63 ms. Time to 50% rise of tension was 88 ms, when all these reflexions have reached nearly 90% of the maximal change.

### No width change in A2 and A4

To interpret the intensity changes, it is important to know if the lateral widths of the reflexions change on activation (see discussion). Therefore, we repeated the experiments placing the detector crossing the meridian. No substantial change in the lateral breadths of reflexions A2 and A4 were observed either during phase one or during phase two (Fig. 4).

### Intensity changes at 18°C

Some experiments were repeated at 18°C. At this temperature, being compared with the results at 4°C, the isometric tension was  $\sim 50\%$  higher and the tension rising

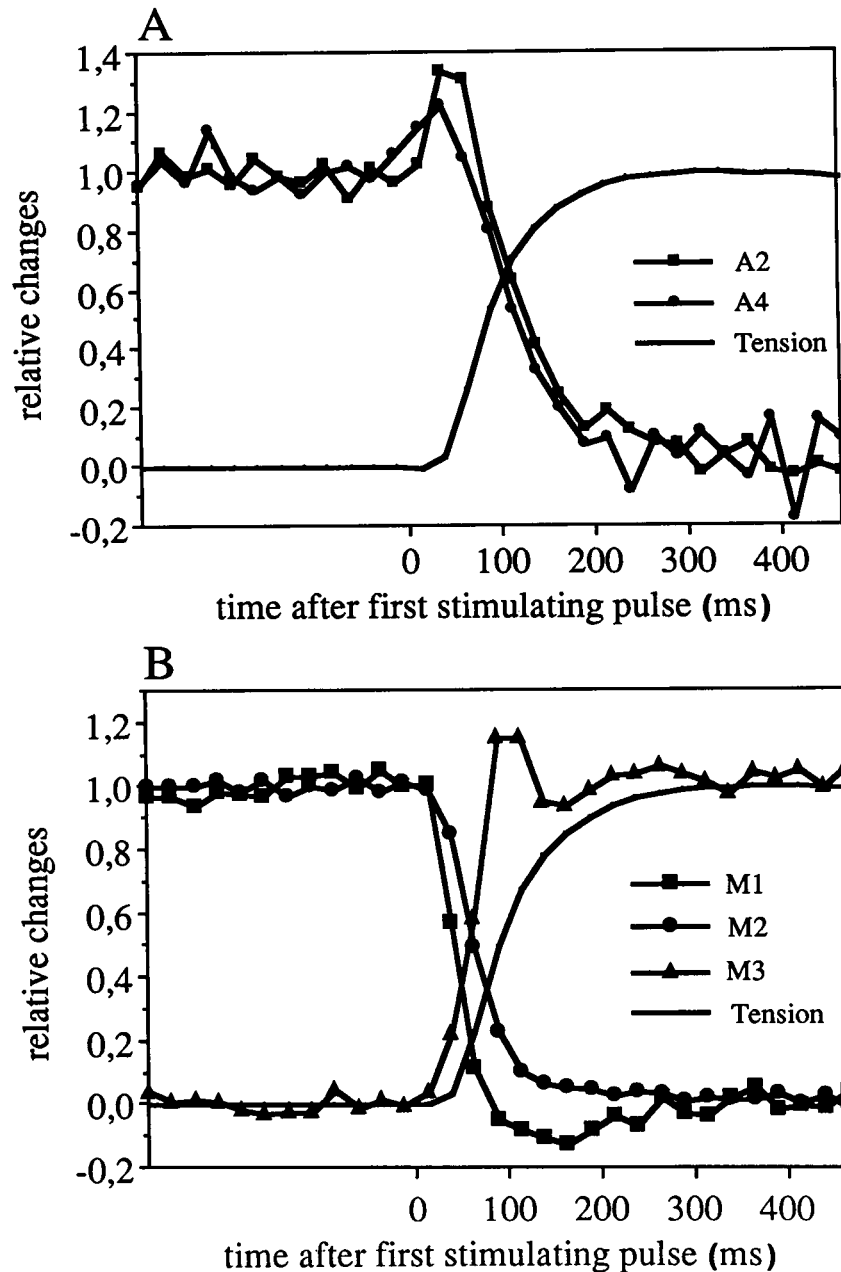


FIGURE 3 Time courses of intensity changes of (A) the thin filament-associated reflexions, and (B) the thick filament-associated reflexions, together with the tension time course. Reflexion intensities after subtracting background are represented in % changes, either the value in the resting state or that during the tension plateau being taken 0 and 100%. The abscissa represents time after first stimulating pulse in ms. Each data point corresponds to a duration of 25 ms. For (A), data were collected from two muscles after 26 contraction cycles in total (Z42, MAY89TN); for (B), from one muscle after 10 contraction cycles (S27, MAY89TN).

phase as well as the tension decaying phase were faster (Fig. 5) by a factor of  $\sim 2$ . Associated with the faster tension time course, the intensity rise in phase one as well as the intensity decay of phase two were also by a factor of 2 faster than at  $4^{\circ}\text{C}$ . It is worth noting that the both at  $4^{\circ}\text{C}$  and at  $18^{\circ}\text{C}$  the A2 intensity did not begin to recover until tension decayed to a level lower than the "shoulder," like the fiber stiffness and the equatorial reflexion intensities recorded from single frog fibers (Cecchi et al., 1991).

## DISCUSSION

The major finding of this study was that two phases were distinguished in the time courses of x-ray intensity changes of A reflexions, A2 as well as probably A4. The phase one was a transient intensity increase, which reached the peak when tension had just begun to rise. This phase was more prominent in A2 than in A4. The phase two was a slow intensity decrease, the time course being almost concomitant with the tension rise. In A2

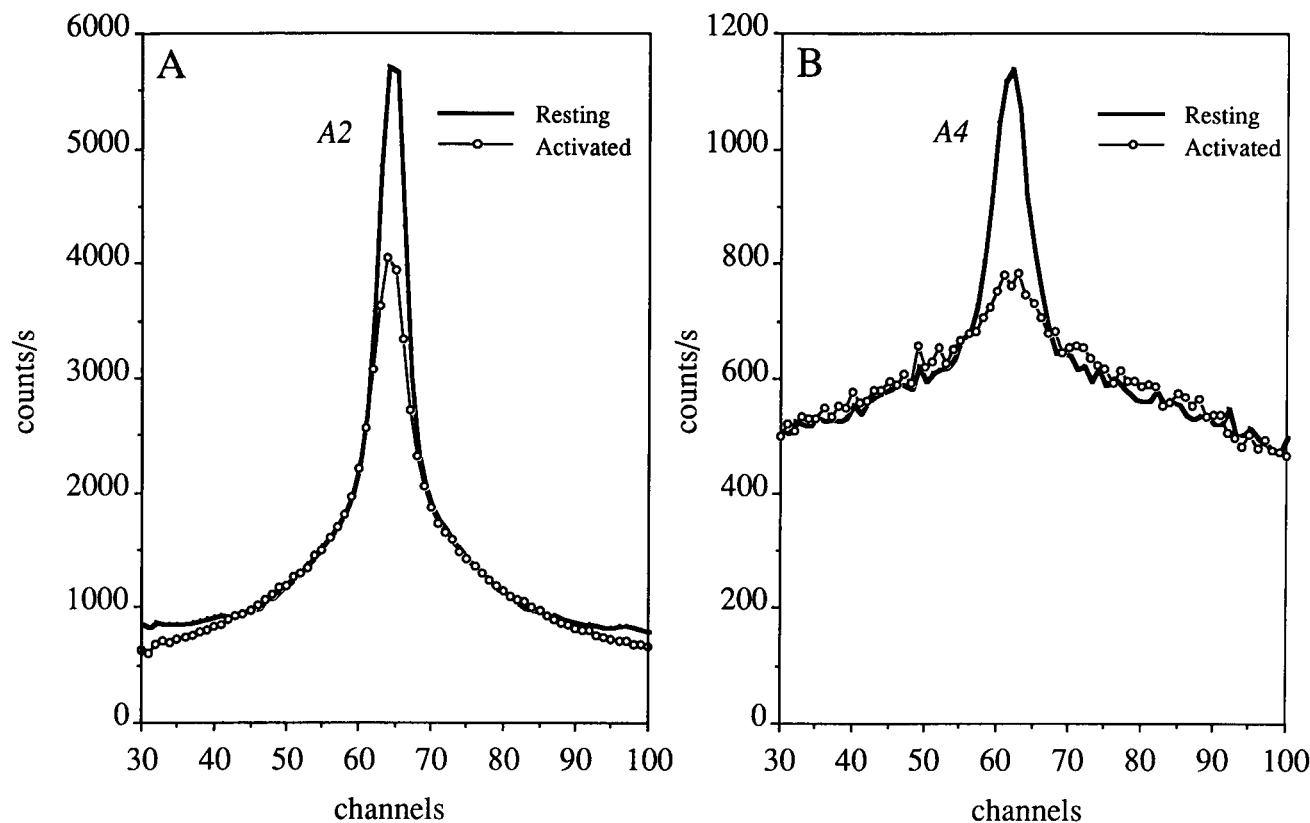


FIGURE 4 Intensity profiles of (A) A2 reflexion, (B) A4 reflexion, across the meridian. In each panel, the upper profile (*plain line*) is from the resting muscle, whereas the lower profile (*open circles*) is from the muscle at the tension plateau. The ordinate, x-ray counts per second. The abscissa, channel numbers. The patterns in (A) were collected from three muscles, 118 contractions in total (W44, SEP89TN), while the patterns in (B) were collected from one muscle after 50 contractions (Z08, SEP89TN).

and A4, this phase brings the intensities into a level lower than the resting level. Both in A2 and A4, neither phase one nor phase two was associated with a lateral broadening of the reflexion.

The meridional A reflexions are the transform of the mass associated with the thin filaments projected onto the filament axis. In other words, the reflexions originate from the periodic alteration of the thickness of the filament. An intensity increase, therefore, would be explained when the periodic alteration becomes more prominent, whereas a more homogeneous mass distribution would result in reduced intensities. Any change in the periodic alteration would affect primarily the intensities of inner reflexions, being consistent with the present results. Intensities would also be affected by various types of disorders or irregularities of the structure, either that of the individual filament or that of the filamentous lattice. The influence of these factors, however, would be more prominent on the reflexions at higher angles than those at lower angles, being not consistent with the present results (see below).

The simplest and most plausible interpretation of phase one, an early intensity increase, is conformational changes of troponin such that troponin becomes more

elongated in the direction perpendicular to the filament axis. First, this intensity increase is observed with furthest inner two reflexions. Reflexions in lower angles are more sensitive to the molecular shape than others. Secondly, the fast time course, reaching the maximum at 40–50 ms after the first stimulating pulse at 4°C, likely being associated with the calcium binding to troponin. This time course is very much the same as that of the intensity increase of the second actin layer-line (Kress et al., 1986; Maéda et al., 1988). The latter has been attributed to structural changes of the thin filament induced by the calcium binding.

Phase two, the delayed intensity decay, likely originates from the binding of the myosin heads (S1) to the thin filament, although an alternative explanation in terms of conformational changes of the troponin-tropomyosin complex is not excluded. The cross-bridge formation would add extra mass to the thin filament between two adjacent troponin levels so that the axial mass modulation becomes less prominent. First, this phase is clearly seen with the two furthest inner reflexions, being consistent with a change in the periodic mass distribution, not likely due to any type of disordering. Second, the time course of the phase is closely associated with the

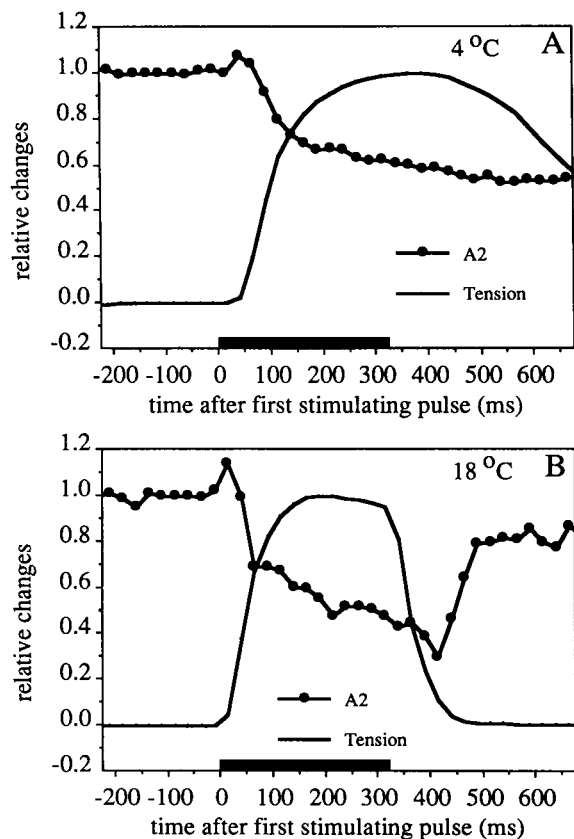


FIGURE 5 The time courses of the intensity change of A2 reflexions together with the tension time course, (A) at 4°C or (B) at 18°C. The ordinate, the reflexion intensity relative to the resting level or the tension relative to the maximum. The abscissa, time after the first stimulating pulse in ms. Stimulation was applied during the period indicated by a thick bar on the abscissa. The duration of each data point, 25 ms. The data were collected, for (A), from three muscles after 118 contractions in total (W44, SEP89TN), or for (B), from one muscle after 40 contractions (T66, SEP89TN).

tension time course both at 4°C and at 18°C. Third, the prolonged reduced intensity level during the tension decaying phase (Fig. 5) is reminiscent of the time courses of the fiber stiffness and the equatorial reflexion intensities which are indicative of cross-bridge attachment (Cecchi et al., 1991). Fourth, the extent of the change in phase two is too large to be attributable to plausible conformational changes of the troponin-tropomyosin complex.

To obtain further supports for the interpretation of phase two, we have recorded x-ray diffraction patterns from chemically skinned rabbit psoas muscles. In the rigor state, both A2 and A4 are substantially weaker than in the relaxed state. Whether or not the cross-bridges formed during tetanus peak are of rigor bridges, this result indicates that extra mass attached to the actin filament could cause the intensity decrease of the troponin reflexions.

For the following reasons, possible contributions of disordering and of irregularities of the structure to the observed intensity changes could be excluded.

(a) If periodic alterations in the mass distribution of individual filaments becomes less regular, being equivalent to a "thermal" disorder of one dimensional crystals (Vainshtein, 1966), then reflexions at higher angle become weaker, those at lower angle being little affected. This is not consistent with the present results which showed substantial changes in the reflexion intensities.

(b) In sarcomere, the thin filaments are axially aligned so that a pair of troponin molecules on individual filaments are at the same level (Ohtsuki et al., 1986). This results in interference between rays diffracted by individual filaments so that meridional reflexion intensities are "sampled" on the meridian, reflexion widths across the meridian being reduced and peaks on the meridian being higher. In case the thin filaments became misaligned on activation of muscle, this sampling effect would disappear, which may result in lateral broadening of meridional reflexions. Our results in Fig. 4 clearly show no change in the lateral widths.

(c) The interference between rays scattered from two halves of the I-band accounts for splitting of a meridional reflexion, especially that at a low angle, into a couple of branches which are closely spaced in the axial direction (Huxley and Brown, 1967; O'Brien et al., 1971; Rome et al., 1973). Misaligning the thin filaments on activation, as discussed above, would diminish this effect too. With or without the interference of this type, the total intensity associated with the branches likely remains unchanged. In the present work, the experimental set-up does not allow us to resolve these branches so that this factor unlikely accounts for the observed intensity changes.

In conclusion, the present study indicated that the calcium activation of the thin filament, particularly the calcium binding to troponin, induces an intensity increase of reflexions A2, presumably as well as A4, while the actomyosin interaction decreases the reflexion intensities. Because the activation and the actomyosin binding cause the intensity changes in opposite directions, and because the two mechanisms have differentiated time courses, the intensities undergo the two phase changes as reported here. The intensity increase of these reflexions would give us information which is exclusively associated with the calcium activation, not with the actomyosin interaction. This is in contrast with another thin filament-associated reflexion, the outer part of second actin layer-line at  $1/19 \text{ nm}^{-1}$ , which is likely influenced not only by the calcium activation but also by the binding of myosin and the two influences are in the same direction (Popp et al., unpublished results).

It would be of particular interest to identify the onset and the termination of the calcium activation by use of the intensity increase of A reflexions. As has been discussed above, the intensity rises of A2 and A4 in phase one reported here likely correspond to the onset of the calcium activation. To identify the terminating time-point of the activation, time courses of A2 or A4 reflex-

ions could be measured from stretched muscles in which the negative contribution of the actomyosin interaction would be reduced.

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