DISASSEMBLY FROM BOTH ENDS OF THICK FILAMENTS IN RABBIT SKELETAL MUSCLE FIBERS

An Optical Diffraction Study

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ABSTRACT We show in this paper that the change of the internal structure of a sarcomere in a rabbit glycerinated psoas muscle fiber can be examined by analyzing the intensity change of the first- and the second-order optical diffraction lines. A unit–cell (sarcomere)-structure model has been applied to the estimation of the length of thick filaments in a muscle fiber while they undergo dissociation. The optical factors, except for the unit–cell-structure factor, hardly changed during the dissociation of the filaments. Our results show that thick filaments dissociate from both ends on increasing the KCl concentration in the presence of 10 mM pyrophosphate and 5 mM $MgCl_2$. Micromolar concentrations of Ca^{2+} suppressed to some extent the dissociation of thick filaments. The disassembly of thick filaments occurred at higher KCl concentrations in the absence of pyrophosphate. There was a correlation between the stability of the thick filament structure and cross-bridge formation, which was induced either by the addition of micromolar concentrations of Ca^{2+} in the presence of Mg-pyrophosphate or by removal of Mg-pyrophosphate.

INTRODUCTION

Although there have been many studies on the structure and physico-chemical properties of thick filaments and many attempts have been made to reconstruct them in solution (Noda and Ebashi, 1960; Huxley, 1963; Kaminer and Bell, 1966; Josephs and Harrington, 1966; Harrington and Himmelfarb, 1972; Katsura and Noda, 1973; Pinset-Härstrom and Truffy, 1979; Reisler et al., 1980 and 1982; Niederman and Peters, 1982), not many studies have been done in a myofibril or a muscle fiber (Hanson and Huxley, 1955; Tawada et al., 1976; Taniguchi and Ishikawa, 1982). This has probably been due to the lack of a suitable method. Electron microscopy, for example, although a powerful tool for studying the structure of myosin filaments, is not suitable for studying the assembly and disassembly process of thick filaments in a muscle fiber, since the structure of filaments may change during fixation and staining of a muscle fiber.

In this study, we have shown that the optical diffraction method makes it possible to examine the length change of myofilaments in a muscle fiber when the optical factors, except for the structure factor of a unit cell, i.e., a sarcomere, do not change. A unit cell structure model proposed by Fujime (1975, 1984; Fujime and Yoshino, 1978) could be applied to the estimation of the length of thick filaments during their dissociation. On the basis of this method, the effects of KCl, PPi,[1] and micromolar concentrations of Ca^{2+} on the dissociation of thick filaments were examined. We concluded that thick filaments dissociate from both ends, maintaining the thickness of the filament constant, which is consistent with the results obtained in vitro (Trinick and Cooper, 1980) and in a myofibril (Ishiwata, 1981*b*). Moreover, we found that micromolar concentrations of Ca^{2+} stabilize the thick filament structure in the presence of PPi.

Preliminary reports on the present work have been presented at the 18th annual meeting of the Biophysical Society of Japan (1980) and the 6th Taniguchi International Symposium on Biophysics (Ishiwata, 1981*a*).

MATERIALS AND METHODS

Muscle Fiber

A bundle of rabbit psoas muscle fibers ~5 mm in diameter was slightly stretched, tied to a glass rod, and stored in 50% glycerol, 0.5 mM sodium bicarbonate, and 5 mM EGTA for more than 3 wk at −20 °C. Just before experiments, a small bundle of fibers, 100–150 μm in diameter and 5 mm in length, was separated under a stereo-microscope at room temperature in the above glycerol solution. The muscle bundle contained ~10 fibers. One end of the bundle was tied with a thin cotton thread to a tension transducer, and the other end to a muscle stretcher. The muscle bundle

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[1] *Abbreviations used in this paper:* EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid; PPi, pyrophosphate.

thus prepared was first immersed for ~20 min in a rigor buffer containing 60 mM KCl, 5 mM $MgCl_2$, and 10 mM Tris-maleate buffer (pH 6.8; adjusted for 0.5 M Tris-maleate solution at room temperature), in an optical cell (5 × 20 × 10 mm), whose temperature was regulated at 10 ± 1°C by a thermomodule (KSM-0671, Komatsu Electronics Co., Hiratsuka, Japan). The pH value of the rigor buffer was decreased to 6.5 on addition of 10 mM PPi (pH 7.0; adjusted for 1 M PPi solution). The dissociation of thick filaments was studied at pH 6.5 whether or not PPi was present. The experiment was started by exchanging the rigor buffer with other soultions. The ATP was purchased from Boehringer Mannheim Co. Ltd. (Indianapolis, IN) and potassium PPi and other chemicals were of reagent grade.

Optical Diffraction (Experimental)

The light beam from a He-Ne laser (632.8 nm; 15 mW nominal; type GLG-5601, NEC Co. Inc., Buckner, MO) was weakly focused on a muscle bundle in the optical cell (a diameter of the laser beam at a muscle bundle, ~0.5 mm) (Fig. 1). Intensities of diffraction lines were measured by changing the position (i.e., the scattering angle 2θ) of a photodiode along a semi-circle (radius 8 cm) on the horizontal plane. The size of a slit in front of the photodiode was 1 × 4 mm. The response of the photodiode against the intensity of incident light was linear. We measured the average value of the intensities at two peaks of each diffraction line. The intensities of diffraction lines were apt to fluctuate, especially under rigor condition (Figs. 3 *a* and 6 *a*). This may be due to disorganization of internal structure in a muscle fiber by repeated stretching of a muscle bundle to measure stiffness.

When the incident angle (ω; 0° at the normal incidence, see the insert in Fig. 1) of the laser beam to a muscle bundle was to be changed, the bundle was horizontally fixed in a cylindrical cell. By rotating the muscle bundle about the vertical axis, the ω-scan intensity profiles of the first- and the second-order diffraction lines were measured.

Optical Diffraction (Theoretical)

According to Fujime (1984; Fujime and Yoshino, 1978), the intensity distribution of the nth-order diffraction line, I_n, of a muscle fiber consisting of many myofibrils with the same radius, R, is expressed as

$$I_n(u_n, \Xi, \Phi) = \Psi(u_n, \Xi, \Phi)(\pi R^2)^2 [2J_1(\Xi R)/\Xi R]^2 L(u_n) |F(u_n)|^2, \quad (1)$$

where (u_n, Ξ, Φ) are the cylindrical coordinates in a reciprocal space corresponding to (x, r, ϕ) in a real space (the x-axis being parallel to the long axis of a muscle fiber), n is the order of diffraction line and $u_n = 2\pi n/L$, where L is the sarcomere length. $\Psi(u_n, \Xi, \Phi)$ is a structure factor of the arrangement of myofibrils in the fiber. J_1 is the first-order Bessel function, $[2J_1(\Xi R)/\Xi R]^2$ is the radial shape factor of a myofibril, $L(u_n)$ is the Laue function, and $F(u_n)$ is the structure factor of a unit cell.

The ω-scan intensity profile is essentially the same as that of the Ξ-scan intensity, although a slight nonlinearity exists in the ω vs. Ξ relationship (Fujime, 1984). The ω-scan profile gives the radial shape factor $\Psi(u_n, \Xi, \Phi)$ $[2J_1(\Xi R)/\Xi R]^2$ of the intensity distribution, thus giving information of fibril arrangement in a cross section of a muscle fiber.

In the present work, no factors other than a structure factor of a unit cell were considered for analyzing the intensity change of the diffraction lines. Thus, the intensity of the nth-order diffraction line was calculated in arbitrary units as follows (see Fujime, 1975):

$$I_n = C|(\pi/Ln_A)F(u_n)|^2, \quad (2)$$

and

$$F(u_n) = (L/\pi n)[n_A \sin(\pi nb/L) + n_I(-1)^n \sin(2\pi na/L) + n_Z(-1)^n \sin(2\pi nc/L)], \quad (3)$$

where n_A, n_I, and n_Z are refractive indices of an A-band, an I-band, and a Z-line, respectively, a and b are the lengths of thin and thick filaments, respectively, and c is the half-width of a Z-line (see Fig. 2). The quantity C, which represents all other factors contributing to the diffraction line intensity (see Eqs. 1 and 2), is assumed to be constant for fixed values of the incident (ω) and the scattering (2θ) angles (or fixed values of u_n, Ξ, and Φ) (see Discussion).

Stiffness

The stiffness of a muscle fiber was measured by a tension transducer similar to that designed by Hellam and Podolsky (1969) and a muscle stretcher, both of which were homemade. The stiffness of a muscle fiber, K, was estimated as follows: A muscle bundle was stretched by $X = 20$ μm (~0.4% of the total length of the bundle) by a stretcher containing a relay and a stop with a micrometer, and the displacement of the lever of the tension transducer, x, was recorded on a chart recorder (QPD54, Hitachi Ltd., Tokyo, Japan). When k denotes the stiffness of the tension transducer, the equation of force balance, $K(X - x) = kx$, holds. In this study, we calculated K/k from the predetermined X and the displacement x. This quantity showed the smallest value under relaxing conditions, the largest under rigor conditions, and an intermediate value under contracting conditions. There are several reports that state that the stiffness is proportional to the number of attached cross-bridges (Kawai and Brandt,

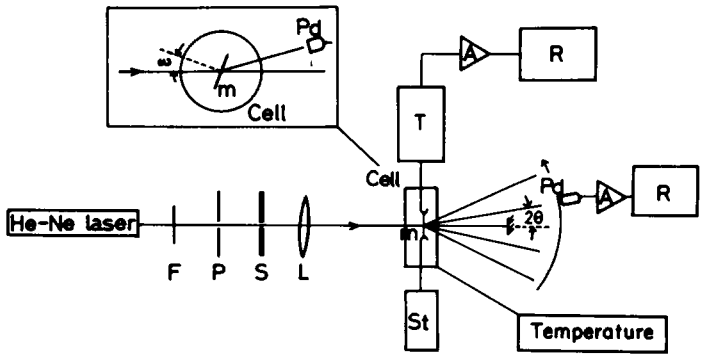

FIGURE 1 This is a schematic illustration of the experimental apparatus for optical diffraction (*top* view). Abbreviations are as follows: *F*, ND filter; *P*, pinhole; *S*, shutter; *L*, lens; *m*, muscle bundle; *Cell*, rectangular or cylindrical (the insert) optical cell; *T*, tension transducer; *A*, amplifier; *R*, pen recorder; *St*, muscle stretcher; *Pd*, photodiode; *Temperature*, temperature regulator and thermometer; 2θ, scattering angle; and ω, incident angle.

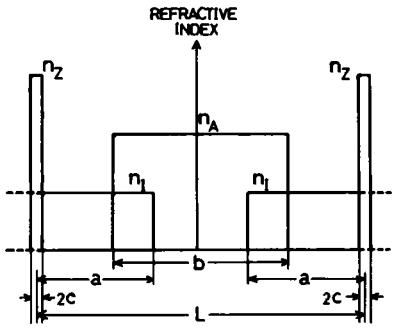

FIGURE 2 This is a schematic illustration of the distribution of refractive index, i.e., protein concentration, in one sarcomere (a unit cell) of a muscle fiber. This distribution was used to calculate the intensity of diffraction lines in Fig. 7. L is the length of a sarcomere; a is the length of a thin filament (1.0 μm); b is the length of a thick filament (originally 1.5 μm and variable); c is the half-width of Z-line (0.05 μm). n_A, n_I, and n_Z are, respectively, refractive indices at A-band, I-band, and Z-line in an arbitrary unit.

1976; Tawada and Kimura, 1984); in this and the following studies, the stiffness value, K/k indicates the number of attached cross-bridges.

RESULTS

Changes of Diffraction Line Intensity and of Stiffness of Muscle Fibers with Increasing KCl Concentration in the Presence of PPi

For each muscle bundle, the diffraction line intensity and the stiffness were alternately measured on increasing the KCl concentration in the presence of 10 mM PPi. Fig. 3 *a* shows that the intensity of the first-order line decreased to nearly zero and then increased to near the original or even a somewhat higher value. The intensity of the second-order line, on the other hand, decreased to nearly zero, then increased and showed a peak, and finally decreased again. Such trends in the intensity changes were reproducibly observed, although the absolute value of the intensity varied from preparation to preparation. It is to be noted here that on addition of Ca^{2+}, the intensity change occurred at a higher KCl concentration.

Fig. 3 *b* shows that the stiffness of the muscle bundle decreased gradually on increasing the KCl concentration. Also, an effect of Ca^{2+} was observed. The diffraction line intensity and the stiffness of muscle fibers did not return to the original values after decreasing the KCl concentration again (not shown).

Changes in ω-Scan Intensity Profiles of Diffraction Lines with Increasing KCl Concentration in the Presence of PPi

In Fig. 3, the diffraction line intensities were measured at the normal incidence of a laser beam to a muscle bundle,

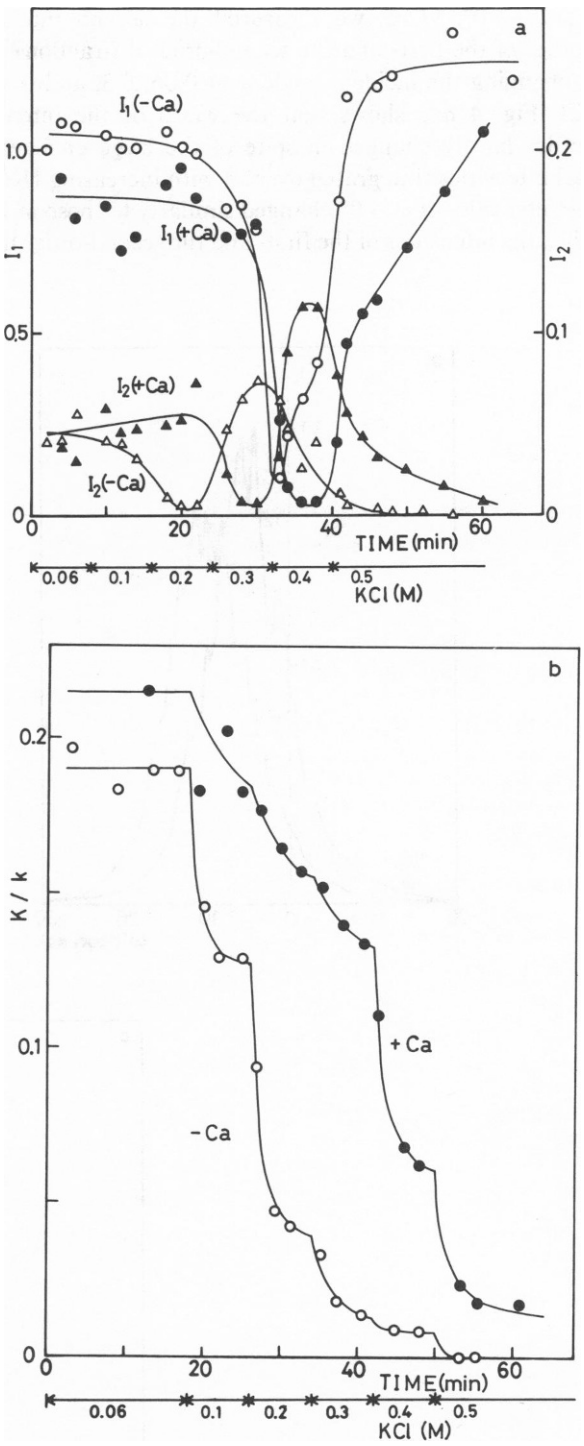

FIGURE 3 This figure shows changes of diffraction line intensities, I_n, in arbitrary units (*a*), and of stiffness, K/k, (*b*), of a muscle bundle with increasing KCl concentration in the presence of PPi. The KCl concentration was increased stepwise and sequentially from 0.06 to 0.5 M at intervals of 8 min, as shown on the abscissa. Note that for the observed quantities at each KCl concentration equilibrium was not attained within 8 min. Different muscle bundles were used in the presence and the absence of Ca^{2+}; bundles of muscle fibers, about 150 μm wide and 5 mm long, were chosen from the same batch of glycerinated muscle. Medium, KCl (variable as indicated on the abscissa), 4.5 mM $MgCl_2$, 10 mM PPi, 10 mM Tris-maleate buffer (pH 6.5) and 100 μM $CaCl_2$ (closed symbols) or 1 mM EGTA (open symbols) at 10°C. Sarcomere length, 2.8 μm.

i.e., $\omega = 0°$. Here, we measured the ω-scan intensity profiles of the first- and the second-order diffraction lines by changing the incident angle ω at 0.06, 0.3, and 0.5 M KCl. Fig. 4 *a–c* shows that the width of the intensity profiles hardly changed in spite of the large changes in total intensities (integrated over ω) with increasing [KCl]. The intensities at $\omega = 0°$ changed similarly to those in Fig. 3, i.e., the intensities of the first- and the second-order lines changed while the shape of the ω-scan intensity profiles was maintained.

Reversibility of the Effect of Ca^{2+}

Fig. 5 shows that the stiffness of a muscle bundle increased on addition of Ca^{2+} and decreased on removal of Ca^{2+}, repeatedly and reversibly. On the other hand, there was practically no change in the intensity of diffraction lines.

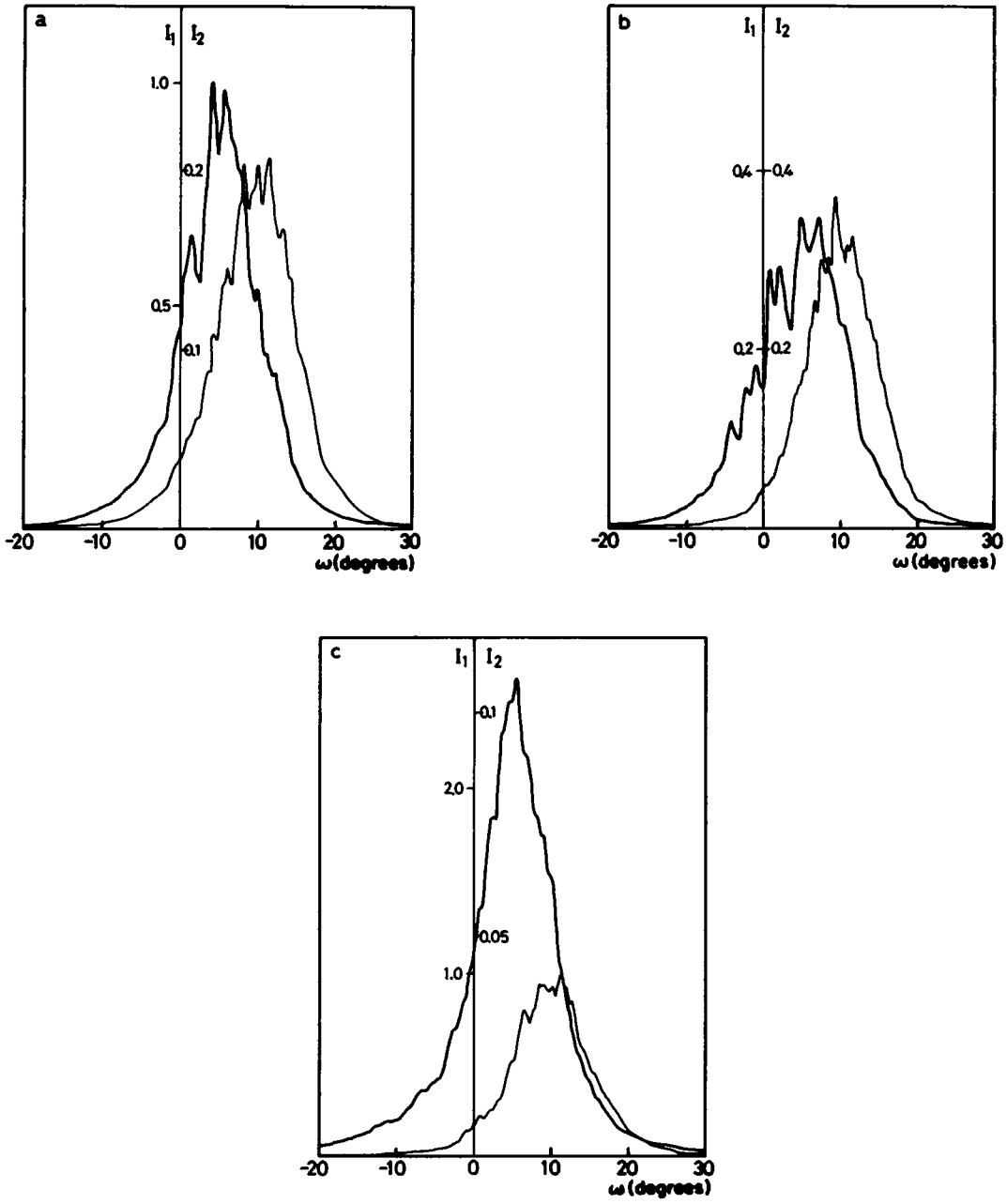

FIGURE 4 Shown here are changes of ω-scan intensity profiles of diffraction lines with stepwise increasing KCl concentration in the presence of PPi. The intensity profiles of the first-order (I_1) and the second-order (I_2) diffraction lines are shown by thick and thin lines, respectively. Ordinates, the intensities normalized to the peak value of the first-order diffraction line in *a*. Abscissa, the incident angle (ω); 0° at the normal incidence. (*a*) in the rigor buffer with 1 mM EGTA; (*b*), in 0.3 M KCl, 4.5 mM $MgCl_2$, 10 mM PPi, 10 mM Tris-maleate buffer (pH 6.5) and 1 mM EGTA; (*c*) the same as in *b* except 0.5 M KCl. Temperature is 10°C. Sarcomere length is 2.7 μm.

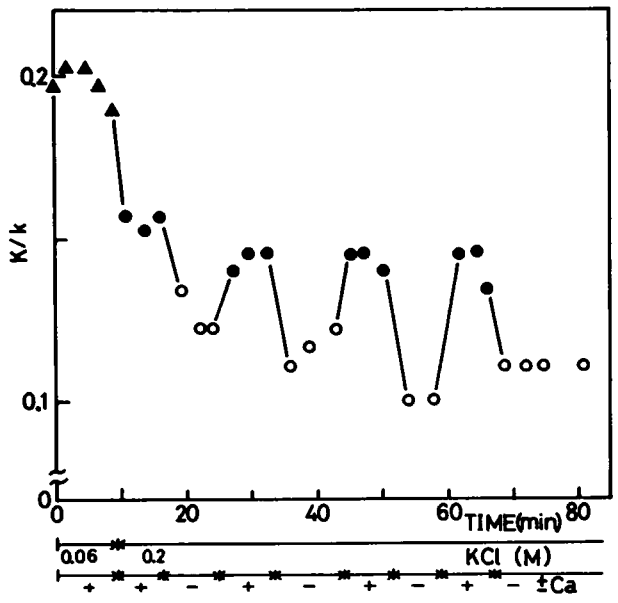

FIGURE 5 This figure illustrates the reversibility of the effect of Ca^{2+} on the stiffness of a muscle bundle. The effects of addition and removal of Ca^{2+} were examined alternately. Medium, 0.2 M KCl, 5 mM $MgCl_2$, 10 mM PPi, 10 mM Tris-maleate buffer (pH 6.5) and 100 μM $CaCl_2$ (●) or 1 mM EGTA (○) at 10°C. ▲, the same as (●) but 60 mM instead of 0.2 M KCl. Sarcomere length is 2.8 μm.

Changes of Diffraction Line Intensity and of Stiffness of Muscle Fibers with Increasing KCl Concentration in the Absence of PPi

In the absence of PPi, the intensity of the first- and second-order diffraction lines was nearly constant up to 0.4 M KCl, slightly decreased at 0.5 M KCl, and considerably decreased at 0.6 M KCl irrespective of the presence or absence of Ca^{2+} (Fig. 6 *a*). The stiffness of a muscle bundle changed similarly (Fig. 6 *b*). These changes occurred at a much higher KCl concentration than in the presence of PPi (see Fig. 3). The difference in the diffraction line intensity (Fig. 6 *a*) or in the stiffness (Fig. 6 *b*) in the presence and in the absence of Ca^{2+} is ascribed to the fact that different muscle bundles were used in the two experiments.

On the Swelling of Muscle Bundles

Because the swelling of muscle fibers would generally affect optical factors, we examined, under an optical microscope, the swelling due to the increase of the KCl concentration from 60 mM to 0.5 M. The radius of a muscle bundle increased by ~10% and ~15% in the presence and in the absence of PPi, respectively. In contrast, corresponding changes for a single fiber were, respectively, ~20% and ~50%. The effect of Ca^{2+} was not detected.

In a single fiber, there was one region where the degree of swelling was much larger than, and another region where it was similar to, that of a bundle. We could observe a network structure surrounding the latter region. Thus, the elastic network present in the space between fibers can be considered to suppress the swelling of a fiber in the bundle. This seems to be a main reason why the degree of swelling was much smaller in a bundle than in a single fiber.

Data Analysis of Optical Diffraction

We calculated the intensity of diffraction lines according to Eqs. 2 and 3 in the case of $n_A = 2n_1 = (2/3)n_Z$ (Fig. 2), which is consistent with the experimental data obtained by

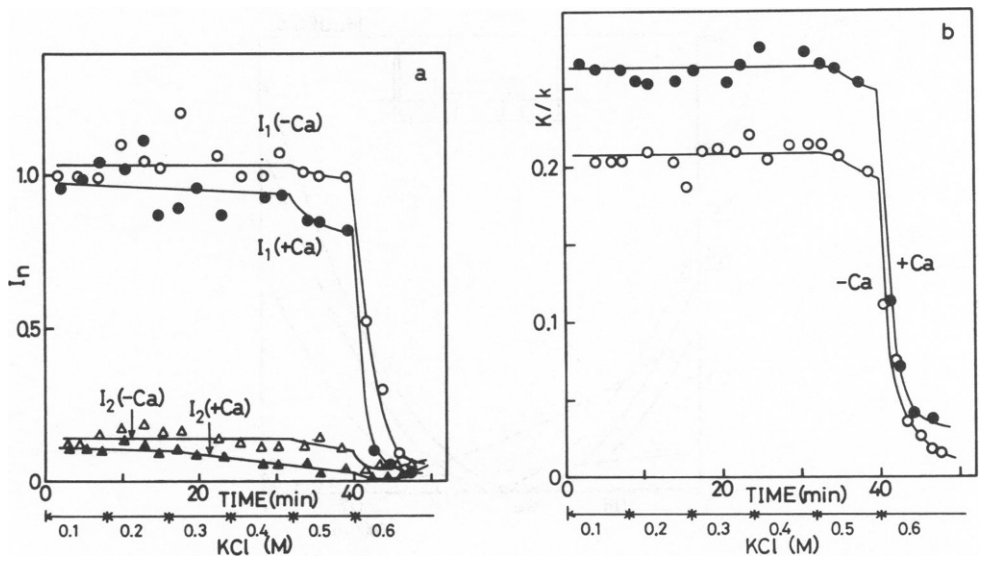

FIGURE 6 Shown here are changes of diffraction line intensities, I_n, in arbitrary units (*a*), and of stiffness, K/k, (*b*), of a muscle bundle with increasing KCl concentration in the absence of PPi. Conditions are the same as in Fig. 3 except for the absence of PPi. The pH value was adjusted to 6.5 under each condition.

Huxley and Hanson (1957) (Fujime and Yoshino, 1978). We neglected the contribution of the M-line because it is small, provided that n_M is equal to n_I and the width of M-line is 0.1 μm (Fujime and Yoshino, 1978). As shown below, the change of the diffraction line intensity obtained is due to the disassembly of thick filaments. We examined the following three models concerning the disassembly mechanism of thick filaments: (*a*) Thick filaments dissociate from both ends, maintaining the thickness and the number of thick filaments unchanged during the disassembly process (only *b*, the length of the thick filament, is variable); (*b*) Thick filaments dissociate from both ends accompanying the proportional decrease of the thickness and /or the number of thick filaments (both *b* and n_A are variable and $b \propto n_A$). This case is optically quite similar to the case that part of myosin molecules dissociated as in model *a* bind to thin filaments uniformly, so that n_I increases (see Discussion); (*c*) The thickness and/or the number of thick filaments decrease, maintaining the length of thick filaments constant (only n_A is variable).

By the optical diffraction method, we can only detect the changes in the distribution of the refractive index, i.e., the protein concentration. Therefore, in the above models *b* and *c*, we can not distinguish between a change in the thickness of each thick filament and a change in the number of thick filaments in an A-band.

The results of our calculation are summarized in Fig. 7 *a–c*. It is obvious that model *c* cannot be accepted, because the intensity of the second-order diffraction lines decreases monotonically on dissociation of thick filaments. There-

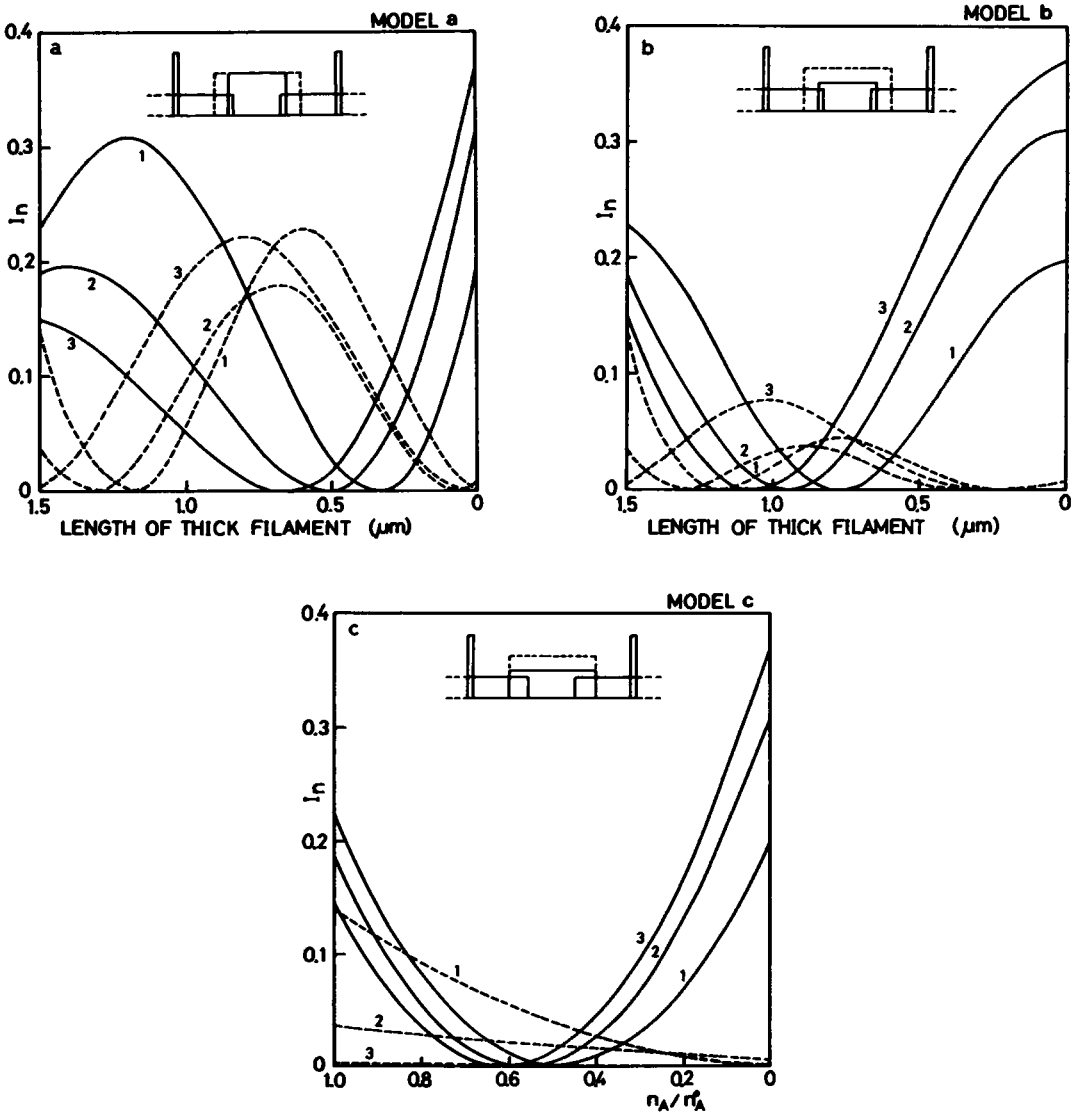

FIGURE 7 This figure illustrates a model calculation of the intensity change in arbitrary units of the first-order (——) and the second-order (----) diffraction lines at sarcomere lengths of 2.4 μm (1), 2.8 μm (2), 3.2 μm (3). (*a*) model *a*; (*b*) model *b* and (*c*) model *c*. The distribution of protein concentration in a sarcomere (Fig. 2) corresponding to each model is schematically illustrated at the top of each figure (dashed line, an intact sarcomere; solid line, a snapshot during the dissociation of an A-band.).

fore, we analyze the experimental results in Fig. 3 according to models *a* and *b*. If a model is correct, the lengths of thick filaments independently estimated from the intensities of the first- and the second-order lines should be equal. This is the criterion we adopted to decide whether model *a* or *b* is more suitable.

The length of thick filaments was determined by comparing the experimental values in Fig. 3 *a* with the theoretical ones in Fig. 7 *a* and *b* at several special points such as a maximum, a half maximum, and a minimum of the intensities of the first- and the second-order diffraction lines. Fig. 8 shows the relationship between the stiffness of a muscle fiber and the length of thick filaments. The following points are to be noted in Fig. 8: First, in the absence of Ca^{2+}, model *a* is appropriate. In the presence of Ca^{2+}, however, it is difficult to judge according to the above criterion which model is better (see Discussion). Second, the stiffness of a muscle fiber in the presence of Ca^{2+} is larger than that in its absence.

The relationship between the length of thick filaments and the KCl concentration is not explicitly shown in Fig. 8. Therefore, it may be worth pointing out that the KCl concentration where the length of thick filaments becomes half of the original one was ~0.3 M in the absence and ~0.4 M in the presence of Ca^{2+}.

It is also to be noted that in Fig. 8, the stiffness in the presence and the absence of Ca^{2+} is compared at the same length of thick filaments. However, the KCl concentrations at each length are different. On the other hand, in Fig. 3 *b* the stiffness was compared at the same KCl concentration but at the different length of thick filaments. In Fig. 5, the Ca^{2+}-effect was examined at the same length of thick filaments and the same concentration of KCl. This is the main reason why the stiffness change by Ca^{2+} in Figs. 3 *b* and 8 looks large when compared with that in Fig. 5.

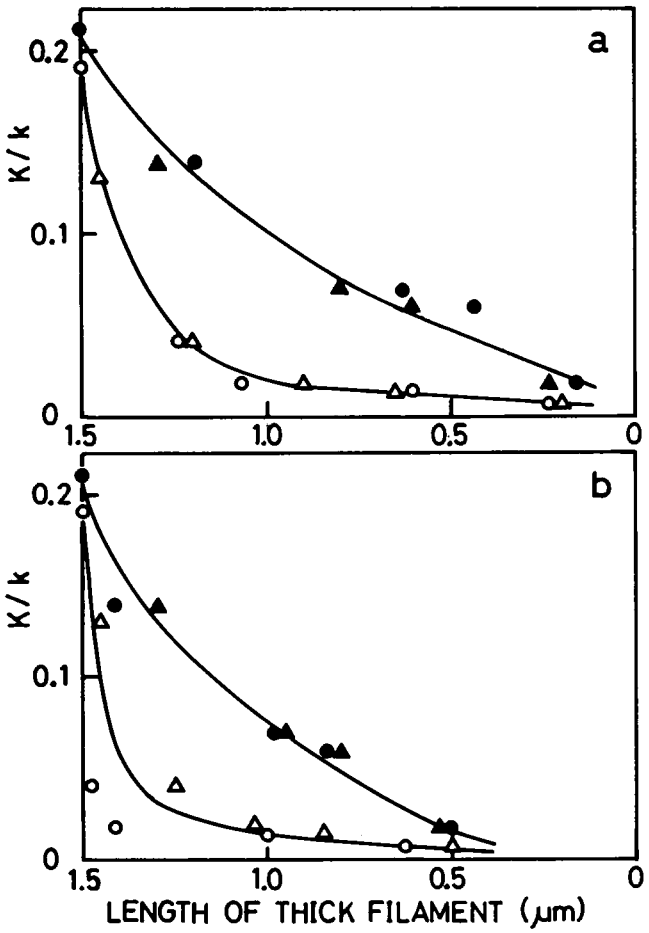

FIGURE 8 Shown here is the relationship between the length of thick filaments and the stiffness of a muscle bundle obtained by comparing the data in Fig. 3 *a* and *b* with the results of model calculation in Fig. 7 *a* and *b* (curve 2). The length was determined from the intensity of the first-order diffraction line (●, ○) or the second-order diffraction line (▲, △) according to model *a* (*a*) and model *b* (*b*). Closed symbols, + Ca^{2+}; open symbols, − Ca^{2+}.

DISCUSSION

Recently many papers concerning optical diffraction of muscle fibers were published (Kawai and Kuntz, 1973; Fujime, 1975 and 1984; Umazume and Fujime, 1975; Paolini et al., 1976; Fujime and Yoshino, 1978; Yoshino et al., 1978; Rieser et al., 1979; Baskin et al., 1979; Rüdel and Zite-Ferenczy, 1979 and 1980; Yeh et al., 1980; Judy et al., 1982; Leung, 1982 and 1983). As pointed out by these authors, the interpretation of the results is generally not simple. In order to fully deduce the structural information from the diffraction pattern, we have to treat a muscle fiber as a three-dimensional phase and amplitude grating. However, when the structural change occurs only in a unit cell, it will be reasonable to analyze the diffraction line intensities according to the structure factor $F(u_n)$. The reason why other factors are considered to be unchanged during the dissociation of thick filaments is as follows: (*a*) Although the intensity changed by a factor of ten during the dissociation process, the change of the width of the ω-scan profile was 10% at most (Fig. 4). If an appreciable change in the lateral arrangement of myofibrils occurred, for example, owing to the swelling of a fiber, it would directly affect the width of the profile, because the width is inversely proportional to the diameter of the bundle of myofibrils that coherently scatters the incident beam. However, the change in the width was actually small, and besides, such a small change may be ascribed to the accidental appearance of the fine structure of the profile. Therefore, very large changes in the diffraction line intensities with distinctly different behaviors in the first- and the second-order diffraction lines, with only small changes in the width of the ω-scan profile (Figs. 3 and 4), cannot be ascribed to the change in the myofibrillar arrangement and/or in the diffractor size. (*b*) Although the swelling of a muscle bundle was observed, its contribution, through the change of R, to the diffraction line intensities is considered to be small, because $[\pi R^2 F(u_n)]^2$ is kept to be nearly constant under the present condition that the diameter of

the laser beam was larger than that of a muscle bundle. Also, the change of $[J_1(\Xi R)/\Xi R]^2$ due to the change of ΞR is estimated to be small. It is reasonable, therefore, to assume that the radial factor $\Psi(u_n, \Xi, \Phi)[2J_1(\Xi R)/\Xi R]^2$ changed little during the dissociation process and that the factor governing the change in the diffraction intensity is ascribed almost fully to the change of $|F(u_n)|^2$.

We estimated the length of thick filaments in a muscle fiber by comparing the experimental results at several special points with the calculated curve (Fig. 7). Although the absolute values of the intensities may be considerably affected by several factors, the position of such special points as a maximum and a minimum of the diffraction line intensities that appeared with the shortening of thick filaments would not be greatly affected. Actually, for example, on increasing a sarcomere length, the relative values of the diffraction line intensities changed nearly in parallel with those estimated from a model calculation, although the absolute values themselves were largely different from each other (Fig. 3 in Rüdel and Zite-Ferenczy, 1980). Thus, the length of thick filaments estimated at those special points will not deviate greatly from the true value (see Fujime, 1984).

The interpretation of the optical diffraction data was supported by SDS gel electrophoresis (under the conditions we examined, proteins originating from thick filaments were selectively released from a muscle fiber; data not shown here; see Ishiwata, 1981*a*) and observations with a phase-contrast microscope (Ishiwata, 1981*b*) or an electron microscope (Higuchi and Ishiwata, 1985).

This study showed that, in the absence of Ca^{2+}, thick filaments in a muscle fiber dissociate from both ends (model *a*). On the other hand, in the presence of Ca^{2+}, it was difficult to judge which model, *a* or *b*, is better (see Fig. 8). The reason for this is as follows.

It was recently shown by an electron paramagnetic resonance study on myofibrils and a physico-chemical study on an in vitro system that the cross-bridge attachment to a thin filament is enhanced either on increasing the Ca^{2+} concentration, on decreasing the KCl concentration or on increasing temperature in the presence of an ATP analog (Ishiwata et al., 1979 and manuscript in preparation; Greene and Eisenberg, 1980). Such properties will not be altered in a muscle fiber either, although an association-dissociation equilibrium between myosin and actin will be shifted towards association because protein concentrations are very high, and the diffusion coefficient of myosin molecules in a muscle fiber will be so small that the reassociation reaction will frequently occur, until myosin molecules are released from the fiber. The dissociation of thick filaments may occur as a net reaction after repeated dissociation and reassociation reactions. Therefore, in the presence of Ca^{2+}, a considerable fraction of dissociated myosin molecules may have become attached to thin filaments, increasing n_I. In this case, the intensity change of diffraction lines may not obey model *a* but rather model *b* even if thick filaments dissociate according to model *a*, because the increase in n_I is nearly equivalent to the decrease in n_A as far as the intensity change of diffraction lines is concerned. To support this interpretation, we made a model calculation by assuming that thick filaments dissociate from both ends as in model *a* and $(Y \times 100)\%$ of dissociated myosin molecules bind to thin filaments uniformly. Fig. 9 shows that as Y increases from 0.5 to 1, the change of the diffraction line intensity becomes similar to that in model *b* (see Fig. 7 *a* and *b*). It is inferred that in the presence of Ca^{2+} 50% of the dissociated myosin molecules are attached to thin filaments (curve 2 in Fig. 9). This interpretation is also supported by the following result: When the temperature is lowered to ~5 °C so that the number of cross-bridges is reduced, model *a* becomes more suitable than model *b* to explain the result in the presence of Ca^{2+} (see Higuchi and Ishiwata, 1985). Therefore, we conclude that even in the presence of Ca^{2+} thick filaments dissociate from both ends, maintaining the thickness of the filament constant as in model *a*. It appears that the function of Ca^{2+} in the presence of PPi is to shift the dissociation curve of thick filaments to a higher KCl concentration; that is, to stabilize the structure of thick filaments. It should be noted that the shift of the minimum position of the second-order diffraction line in Fig. 3 *a* indicates the shift of the dissociation curve, because the minimum position corresponding to ~1.3 μm of the thick filament length is independent of the models (see Fig. 7 *a*, *b*, and 9).

In the absence of PPi, both diffraction line intensity and

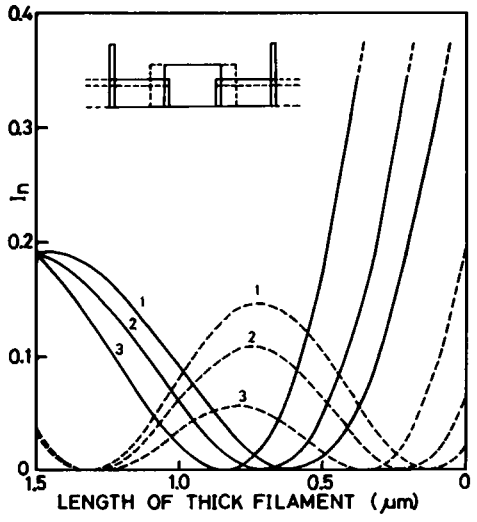

FIGURE 9 This figure shows a model calculation of the intensity change in arbitrary units of the first-order (——) and the second-order (---) diffraction lines at a sarcomere length of 2.8 μm. The refractive index of I-band, n_I, is assumed to change according to the following equation: $n_I = [1 + Y(1.5 - b)]\, n_A/2$, where Y is a proportion of myosin molecules bound to thin filaments after dissociation (0.2 [1], 0.5 [2], 1.0 [3]), b is the length of thick filaments corresponding to the abscissa, and n_A is a refractive index at the A-band. The inserted illustration is the same as in Fig. 2.

stiffness began to decrease at around 0.5 M KCl irrespective of the Ca^{2+} concentration (Fig. 6 *a* and *b*). Phase-contrast micrographs show that the density at a central part of each A-band begins to decrease at nearly the same KCl concentration as above, whereas the density in each overlap region of A-bands remains hardly changed (micrographs not shown; Hanson already pointed out that a central part of an A-band was preferentially removed under high ionic strength without PPi (Hanson and Huxley, 1955), which suggests that cross-bridges remain attached to thin filaments even if the structure of thick filaments is disorganized. It is likely that disorganization occurs over the whole thick filament almost simultaneously under rigor conditions. In the absence of PPi, the KCl concentration at which thick filaments dissociate was 0.5–0.6 M (Fig. 6), which was much higher than that in vitro, namely, ~0.2 M (see Kaminer and Bell, 1966). This stabilization of the thick filament structure will be mainly ascribed to cross-bridge formation.

The effect of micromolar concentrations of Ca^{2+} on the stabilization of the thick filament structure in the presence of PPi will be interpreted as follows: (*a*) As judged from the strong stabilization of the thick filament structure under a rigor condition, the factor most responsible will be the cross-bridge formation promoted by binding of Ca^{2+} to troponin. An attractive idea is that the cross-bridge formation allosterically strengthens the binding between myosin molecules. An alternative is that the probability of reassociation among tail portions of myosin molecules increases because cross-bridges are still there. The reason why the Ca^{2+} effect was not observed in the absence of PPi may be that the cross-bridges were fully formed irrespective of the Ca^{2+} concentrations. (*b*) Another possibility is that the disassembly of thick filaments is independent of cross-bridge formation and regulated by direct binding of Ca^{2+} to myosin.

This second possibility could be examined by using a muscle bundle stretched until overlap between thick and thin filaments disappears (sarcomere length > 3.6 μm). Unfortunately, reliable results could not be obtained at a sarcomere length >3.6 μm because the structure of the muscle fiber was distorted. However, we could examine the dependence on sarcomere length, from 2.4 to 3.2 μm, of the dissociation rate of thick filaments in the subsequent paper (Higuchi and Ishiwata, 1985). The results showed that the Ca^{2+}-effect became small with increasing sarcomere length. Also, there was no effect of Ca^{2+} on dissociation by KCl of synthetic myosin filaments in solution, which was studied by a turbidity change (Higuchi, H., and S. Ishiwata, unpublished work). These results are not favorable to the second interpretation but do not conflict with the first one.

It has become possible through the present work to investigate the assembly and disassembly process of thick filaments and the concomitant change of physiological properties in skeletal muscle fibers. We expect that the line of approach reported here will be fruitful for examining the structural stability of thick and thin filaments and clarifying the molecular mechanism of the assembly and the maintenance of these filaments in a muscle fiber.

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