Approaches to physical fitness and sports medicine through X-ray diffraction analysis of striated muscle

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Abstract X-ray diffraction analysis is a method to obtain information about periodically repeated structures. When striated muscle is irradiated with X-ray, many of the reflections and the layer lines, which convey information about molecular structures within the muscle fiber, are obtained without chemical modification. Two of the strong equatorial reflections, 1,0 reflection arising from a thick filament array and 1,1 reflection arising from a thick and thin filament array appear on the equator giving information about distance and mass distribution on a radial plane. Thus a 1,1/1,0 intensity ratio is a good index of radial distribution of myosin heads. Meridional reflections and layer lines, such as a myosin reflection of the 14.3 nm repeat and myosin or actin layer lines, give information about longitudinal arrangement of the molecules. Since they are affected by the movement of the myosin heads and the shifting motion of troponin-tropomyosin on the actin filament, they can be used to detect conformational changes of contractile and regulatory systems upon muscle activation. The X-ray diffraction method has been applied to yield fruitful results for many problems such as muscle atrophy by disuse, functional modulation by myosin regulatory light chain phosphorylation, differential characteristics of slow and fast skeletal muscle structure, and pathogenesis of some types of the familial myopathy. The approach using X-ray diffraction analysis will continuously serve as a potent tool for resolving problems in the field of physical fitness and sports medicine.

Keywords: X-ray diffraction, skeletal muscle, disuse, phosphorylation, myopathy

X-ray diffraction analysis of striated muscle

By irradiating striated muscle with a light beam, one can record a diffraction pattern on a light-sensitive recording plate such as film (Fig. 1). The pattern is symmetrical about the point of the incident light beam (origin). When a monochromatic X-ray is used as the light beam, the diffraction pattern consisting of reflections and layer lines arising from periodic structures whose period is comparable to the wavelength of the X-ray, that is, on the order of angstroms, appears. The distance of each reflection or layer line from the origin is inversely proportional to the period of a repeated structure (Bragg’s Law) with its intensity reflecting the electron density and volume, and therefore the mass of the repeated structure. Thus, X-ray diffraction analysis is available for obtaining information about the molecular organization of sarcomeres in a physiological state without requiring any chemical modification of the specimen.

Equatorial reflections

As an example, when a striated muscle specimen is set vertically on an X-ray path, hexagonally packed thick filaments in a sarcomere cause a series of strong reflections along the horizontal line (equator) symmetrically extending from the origin (Fig. 2). Each reflection along the equator (equatorial reflection) represents a set of equi-spaced parallel planes passing through the thick and thin filaments in the sarcomere. One can deduce the spacing of

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the planes in the sarcomere from the separation between the corresponding pair of reflections symmetrical about the origin at a given distance between the specimen and the recording plate, and the wavelength of an X-ray. The pair of equatorial reflections nearest the origin is called the 1,0 reflection (Fig. 2, “1,0”), which represents the set of planes of the widest separation (Fig. 3, upper). This set of planes is named the 1,0 plane and has a spacing of 35-41 nm in vertebrate striated muscle. Hereafter, because of the reciprocal relationship between the spacing in real sarcomeres and the distance from the origin on the diffraction pattern, the position on the diffraction pattern is referred to as the reciprocal number of spacing in real space. Accordingly, the 1,0 reflection of vertebrate striated muscle appears at 1/35-1/41 nm\(^{-1}\) on the equator.

The set of reflections appearing in the second nearest position is called the 1,1 reflection (Fig. 2, “1,1”), which arises from a set of planes shown in the lower part of Fig. 3. Unlike the 1,0 plane on which only thick filaments are aligned, both thick and thin filaments are aligned on the 1,1 plane. This makes the relative intensity ratio of the 1,1 reflection to 1,0 reflection (1,1/1,0) a useful index of the radial distribution of myosin heads in the sarcomere structure, because the reflection intensity reflects the mass of a periodic structure. When myosin heads are near the myosin rod, the mass of myosin heads contributes to the mass on the 1,0 plane (Fig. 3, left) intensifying the 1,0 reflection, but when myosin heads swing apart from the myosin rod toward the thin filament to interact with actin molecules, the mass of myosin heads shifts to contribute to the mass on the 1,1 plane (Fig. 3, right), increasing the intensity of the 1,1 reflection and decreasing the intensity of the 1,0 reflection to increase the 1,1/1,0 ratio. Thus, one can estimate the progress of interaction between myosin heads and actin in a sarcomere from the equatorial reflections of striated muscle.
Meridional reflections and layer lines

The vertical line extending from the origin on the diffraction pattern is called the meridian. Along the meridian, reflections arising from sarcomeric proteins that are arranged periodically along the long axis of myofilaments are observed symmetrically about the origin (Figs. 2 and 4). Similar to the equatorial reflections, these reflections appear at the positions inversely proportional to the spacing of the periodic structure. At 1/14.3 nm⁻¹ on the meridian, for instance, the repetition of massive myosin heads positioned along thick filaments causes a strong reflection (Fig. 2, “M3”). In addition, there is a high order of repeats of 42.9 nm spacing caused by the helical arrangement of the heads (Figs. 2 and 4). Such a helical arrangement of the mass deforms the reflections to a set of layer lines, which align horizontally in parallel with the equator. The horizontal intensity distribution of each layer line conveys information about the radial distribution of the helically arranged mass along the helix (radius of gyration).

The reflections and layer lines from each periodic structure appear in a series of positions where Bragg’s reciprocal law is satisfied. They are designated in numerical order from the origin. The helical arrangement of myosin heads forms a set of layer lines in the resting condition (myosin layer lines; MLLs). The first myosin layer line is found at 1/42.9 nm⁻¹ (“MLL1” in Fig. 2), the second at 1/21.5 nm⁻¹ (“MLL2” in Fig. 2), the third at 1/14.3 nm⁻¹ (“MLL3” in Fig. 2), and so on. The double helix formed by actin molecules on a thin filament forms a set of actin layer lines (ALLs). The first actin layer line is found at 1/36 nm⁻¹ (“ALL1” in Fig. 2), the second at 1/18 nm⁻¹ (“ALL2” in Fig. 2), and so on. When myosin heads stereospecifically interact with actin molecules, for example, in the absence of ATP (rigor state; Fig. 2 left), the alignment of myosin heads shifts from the intrinsic helical order represented by MLLs in resting state (Fig. 2 right) to conform to the helical arrangement of actin molecules represented by ALLs, causing a marked shift of first layer line position from 1/42.9 nm⁻¹ (MLL1) to 1/36 nm⁻¹ (ALL1). Modifying the higher order repeat of the helix, the position of the regulatory troponin-tropomyosin complex winding around the thin filament particularly affects the second ALL (ALL2). This allows the shifting motion of the troponin-tropomyosin complex upon activation of the muscle to be monitored. In addition to the above specific movement of sarcomeric proteins, the reflections and layer lines are highly sensitive to any deterioration in the periodicity of the sarcomeric structure. Structural deterioration is considered to have significant importance particularly in the fields of physical fitness and sports medicine as described in the next section.

Structural change of skeletal muscle by disuse

Disuse of skeletal muscle impairs contractility. Although impaired contractility is generally considered to be primary due to degradation of sarcomeric proteins triggered by a loss of stimulation from the nervous system and gravity¹⁻³), a decrease in force per muscle cross-sectional area (specific force) in disused muscle has also been reported. Several researchers have speculated the mechanism of the decrease in specific force, but its structural basis has not yet been clarified. In the rat *m.soleus* immobilized by a cast, Udaka et al.⁴) (Fig. 5) found an increase in the spacing of the thick filament lattice and alteration of length dependency of Ca²⁺-activated force development with a decrease in the level of one of the sarcomeric proteins, connectin/titin. This protein anchors thick filaments to the Z-band and is hypothesized to be a determinant of the spacing of the thick filament lattice and length-dependent activation of the force⁵). Based on
reports that the increased distance between thick and thin filaments has been speculated to decrease the attachment rate of myosin heads to actin by Cazorla et al.6) and Fukuda et al. 7), a decrease in specific force by immobilization can be explained by increased lattice spacing and an altered length-dependent activation mechanism due to a decrease in connectin/titin.

As another way to observe the effects of immobilization on skeletal muscle structure, the denervation procedure is effective. Although atrophy of skeletal muscle8,9) and remarkable increase of expression of ubiquitin ligase system10) has been established to be apparent at around 3 days after denervation treatment, Germinario reported that single skinned rat skeletal muscle fiber exerted larger tension at 1µM Ca2+ a day after denervation compared to control fiber without denervation treatment 11). Sakakima et al.12) also reported, using an electron microscope, that disorder in the Z-band structure started a day after denervation treatment. Furthermore, earlier than those changes, Yokomizo13) detected changes in the sarcomeric structure by obtaining an X-ray diffraction pattern from rat m. tibialis anterior with denervation treatment of the sciatic nerve. He reported a decrease in the intensity of myosin-based meridional reflections at 1/14.3 nm⁻¹ (“M3” in Fig. 2, red arrows in Fig. 6) started from as early as 0.5 days after denervation. Moreover, the 1,0 spacing on day 0.5 concomitantly decreased with the suggestion of enhanced development of specific force and increased Ca²⁺ sensitivity of sarcomeres evidenced as a larger tension development at low Ca²⁺ concentrations. This study showed that X-ray diffraction analysis can detect a subtle structural change at an early stage, which modulates contractile interaction between myosin heads and actin, and triggers a series of degradation processes.

**Effect of phosphorylation of myosin regulatory light chain**

Each myosin head has a regulatory light chain. In smooth muscle, phosphorylation of the regulatory light chain mainly regulates contractile activity, as the tropinin-tropomyosin complex does in striated muscle. Even so, phosphorylation of the myosin regulatory light chain in striated muscle has been reported to potentiate force development during prolonged or repeated contractions of skeletal muscle14-16) and characterize fatigued state of muscle17) induced by activation of myosin light chain kinase by Ca²⁺-bound calmodulin. Moreover, the endogenous phosphorylation level of the myosin regulatory light chain is proposed to determine the kinetics of force development of myocardium18) and modulate ATPase activity in the resting state of skeletal muscle possibly to increase thermogenesis to adapt to the environment19). Therefore, phosphorylation of the myosin regulatory light chain is now regarded as an indispensable modulator of striated muscle performance in a wide range of a given situation.

As a structural base for the modulation mechanism, phosphorylation of the myosin regulatory light chain has been proposed to change the myosin head structure from the one tightly attached to the myosin rod to that loosely linked to the thin filament. Evidence supporting this hypothesis was obtained initially by electron microscopy of an isolated thick filament20-22), and then confirmed by X-ray diffraction analysis of tarantula striated muscle23) and mouse cardiac muscle fibers24). The X-ray diffraction pattern of the muscle with phosphorylated myosin regulatory light chains was found to show a high 1,1/1,0 ratio, strongly supporting the hypothesis.

**Relationship between myosin structure and contraction kinetics**

Although equatorial reflections give much information about behavior of myosin heads and actin, we can obtain more direct knowledge about molecular structures by observing layer lines because they contain information about order, gyration radius, and alignment of the molecules.
forming the helix structure. For example, the intensity of MLL1 can be changed by manipulating the experimental solution to mimic or reproduce physiologically important states. When a mammalian skeletal muscle fiber is treated by crosslinking agents such as N-phenylmaleimide or cooled, MLL1 is weakened; and the peak position, which is reciprocally related to the gyration radius of the helix formed by the myosin heads, shifts toward the meridian. Since crosslinking and cooling shift the chemical state of myosin heads from the M-ADP-Pi state to the M-ATP state, the observed decrease in the intensity and peak shift of the MLL1 is considered to indicate that myosin heads in the M-ATP are released from the rod and disordered. Furthermore, based on the observation of MLL1 in the M-ADP and the M state in overstretched fast skeletal muscle fibers, Xu et al. concluded that myosin heads form a rigid helix only when they are in the M-ADP-Pi state. These studies provide us with a framework for analysis of MLLs and information about the distribution of ATP hydrolysis intermediate, which could be a key to linking contraction kinetics and molecular structures of myosin as described in the next section.

**Structural difference between slow and fast skeletal muscles**

Slow skeletal muscle is structurally different from fast skeletal muscle in the composition and arrangement of sarcomeric components, such as the Z-band structure and M-line composition. In X-ray diffraction analysis, although the general characteristics of diffraction patterns from slow skeletal muscle are similar to those from fast skeletal muscle, a suggestion of some difference in the arrangement of thin and thick filaments, elastic modules determining the lattice spacings, transition rate of myosin heads upon activation, and nucleotide-binding properties have been reported. We recently compared MLLs in fast and slow skeletal muscle, in detail, and found that MLL1 in resting condition shows a lower intensity, at approximately 1/20 nm\(^{-1}\) from the meridian along the equatorial direction, in slow skeletal muscle fibers than in the fast skeletal muscle fibers of a rat leg when those muscles were stored in an ATP-deficient solution to suppress excess myosin light chain kinase activity. Considering that the intensity peak at approximately 1/20 nm\(^{-1}\) in mammalian muscle can be explained by the sampling effect due to the orderly arrangement of myosin heads in the radial plane of the hexagonal myofilament lattice, the observed differences in the X-ray diffraction pattern are likely caused by the disorderly arrangement of myosin heads in the slow skeletal muscle. When these fibers were stored in a solution containing ATP and phosphatase inhibitors to raise the endogenous myosin light chain kinase activity, the intensity distribution of the MLL1 in fast skeletal muscle fibers was not significantly different from that of slow skeletal muscle fibers stored in

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**Fig. 6** X-ray diffraction pattern obtained from *m.tibiaris* anterior of rats with or without denervation treatment. A: Control (without denervation), B: Control (without denervation, polyethylene glycol and butanedionemonoxime were added to compensate lattice swelling by demembranation), C: 0.5 days after denervation treatment (polyethylene glycol and butanedionemonoxime were added), D: 1 day after denervation treatment (polyethylene glycol and butanedionemonoxime were added). Arrow heads indicate 1/14.3 nm\(^{-1}\) meridional reflections of myosin (“M3”). Intensities in the diffraction patterns were represented by pseud color system so that intensities decrease from red to dark blue. ©Yokomizo S. 2012. Originally printed in Master’s thesis. School of Physical Education, Tokai University.
and increases in the intensity of the ALL2 and ALL6 upon activation were smaller. Based on these results, they concluded that the D286G actin mutation suppressed tropomyosin movement, allowing limited interaction between actin and myosin.

Another valuable strategy for studying the pathogenesis of inherited diseases is X-ray diffraction analysis of protein-exchanged striated muscle specimens. A merit of this method is that the primary effect of protein mutation on the muscle structure can be observed without causing a secondary effect such as functional disability or compensatory response of the host animal. To study cardiomyopathy caused by troponin mutation, we applied this method, in which endogenous wild troponin in cardiomyocytes was replaced with exogenous mutant troponin according to the method originally developed by Hatakenaka et al.\textsuperscript{42}. We studied the effects of two types of mutants, K247R and E244D of the cardiac troponin T-subunit, which is considered to mediate the regulatory signals from C- and I-subunits to tropomyosin and actin. The functional and clinical phenotypes for both mutants are quite similar - increased contractility of the cardiac muscle and hypertrophic cardiomyopathy\textsuperscript{43,44}. However, in the X-ray diffraction experiment using mutant T-subunit reconstituted cardiomyocytes, from which I- and C-subunits were intentionally removed, the myocytes exhibited different characteristics. The myocyte reconstituted with the E244D T-subunit mutant showed a significant increase in the equatorial 1/1,1/0 intensity ratio compared to before reconstitution of the mutant subunit T. The myocyte reconstituted with the K247R T-subunit mutant, on the other hand, showed no detectable change in the 1,1/1,0 ratio\textsuperscript{45}. This suggests that K247R causes hypercontractil-
ity via a modified interaction with the troponin I-subunit, while E244D causes hypercontractility by affecting the interaction between the T-subunit and tropomyosin and/or actin independently of interaction with the I-subunit.

Recently, several polymorphisms of sarcomeric proteins, such as R577X of α-actinin 3, have been suggested to have significant effects on the performance of skeletal muscle. X-ray diffraction analysis would be a potent method to clarify the structural effects of such gene modification at molecular levels.

Concluding remarks

The X-ray diffraction method is a powerful tool in the fields of physical fitness and sports medicine for studying molecular modifications induced by diverse levels of perturbations. Further progress of X-ray diffraction analysis will give new insights into important issues in the field.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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