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Neutron diffraction measurements of skeletal muscle using the contrast variation technique: Analysis of the equatorial diffraction patterns

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ABSTRACT

Among various methods for structural studies of biological macromolecules, neutron scattering and diffraction have a unique feature in that the contrast between the scattering length density of the molecules and that of the solvent can be varied easily by changing the D_2O content in the solvent. This "contrast variation" technique enables one to obtain information on variations of scattering length density of the molecules of interest. Here, in order to explore the possibilities of the contrast variation technique in neutron fiber diffraction, neutron diffraction measurements of skeletal muscles were performed. The neutron fiber diffraction patterns from frog sartorius muscles were measured in various D_2O concentrations in the relaxed state where no tension of muscle is produced, and in the rigor state where all myosin heads of the thick filaments bind tightly to actin in the thin filaments. It was shown that in both states, there were reflections having distinct contrast matching points, indicating a variation in the scattering length density of the protein regions in the unit cell of the muscle structure. Analysis of the equatorial reflections by the two-dimensional projected scattering length density map calculations by Fourier synthesis and model calculations provided the phase information of the equatorial reflections, with which the projected scattering length density maps of the unit cell of the hexagonal filament array in both states were calculated. The analysis showed that the scattering length density of the thick filament region was higher than that of the thin filament region, and that the scattering length density of the thick filament backbone changed as muscle went from the relaxed state into the rigor state.

1. Introduction

Among various methods for structural studies of biological macromolecules, neutron scattering and diffraction provide unique information that cannot be obtained from other methods. Because of a large difference in the scattering length density of water and heavy water (-0.00562×10^{-12} cm Å^{-3} for H_2O and 0.064×10^{-12} cm Å^{-3} for $^2H(=D)_2O$) due to the difference in the scattering length of hydrogen (-0.3742×10^{-12} cm) and deuterium (0.6671×10^{-12} cm), it is possible to vary widely the scattering length density of solvent by changing the fraction of D_2O in the solvent (Jacrot, 1976). This "contrast variation" technique has found wide applications in small-angle neutron scattering (SANS): SANS studies of biological macromolecules provide information on internal structures of biological macromolecules, such as scattering length density variations within a protein molecule, and shape information of individual components within a complex consisting of multiple components (Jacrot, 1976; Kneale et al., 1977; Stuhrmann and Miller, 1978; Timmins and Zaccai, 1988). This technique has also been employed in low-resolution neutron crystallography, which provides information on individual components in a very large macromolecular complex such as nucleic acid regions within viruses (Timmins et al., 1994) and nucleosomes (Bentley et al., 1981), and on disordered components in complexes such as lipid moiety in lipoproteins (Timmins et al., 1992; Hermoso et al., 1997) and detergent moiety in membrane proteins (Roth et al., 1989, 1991; Pebay-Peyroula et al., 1995; Snijder et al., 2003; Prince et al., 2003). The contrast variation technique is thus applicable to any system as long as the measurements are at low resolution where scattering and diffraction arise from the contrast, the difference between the scattering length density of the particles of interest and that of the solvent which is considered to be a homogeneous continuum. It is equally applicable to partially ordered systems such as stacked membranes (Hauss et al., 1990) and oriented filamentous samples (Hulmes et al., 1980). It is therefore of interest to see what information can be obtained from the application of the contrast variation technique to neutron fiber diffraction as there are many filamentous systems of biological macromolecules including nucleoprotein complexes such as chromatins and filamentous viruses, and microtubules and muscles. We applied the contrast variation technique to neutron fiber

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diffraction measurements of vertebrate skeletal muscles, as a typical example of a filamentous system.

Skeletal muscles contain two major components, the thick filament containing myosin, connectin/titin, and C-protein, and the thin filament containing actin, troponin, tropomyosin, and nebulin. The thick filaments are axially oriented, laterally ordered into a hexagonal array and interdigitating with an array of the thin filaments. Muscles provide characteristic fiber diffraction patterns that come from these helical filaments. Structural changes during muscle contraction have been extensively studied by X-ray fiber diffraction (Huxley, 2004; Harford and Squire, 1997; Wakabayashi and Yagi, 1999; Squire and Knupp, 2005; and references therein). Neutron diffraction measurements of muscles were done during 1970s in pioneering works by Worcester et al. (1976), and Rodger et al. (1977), but have not been explored further since then. Here we measured the neutron fiber diffraction patterns from frog skeletal (sartorius) muscles in the relaxed state where no tension of the muscle is produced, and in the rigor state where all the myosin heads of the thick filaments bind tightly to actin in the thin filaments. The measurements were done in various D_2O concentrations of the solvent. Dependence of the equatorial and meridional reflections on D_2O concentration was investigated. In both states, there were reflections having distinct contrast matching points, indicating a distribution of different scattering length densities within the protein regions in the unit cell of the muscle structure. Particular attention was paid to the equatorial reflections, which arise from the hexagonal arrays of the thick and thin filaments in the A-band of the muscle sarcomere and reflect the scattering length density distribution projected onto a plane perpendicular to the fiber axis of these filaments. Analysis of the equatorial reflections showed that the phase information for the equatorial reflections may be obtained using the contrast variation series, that there are differences in the average scattering length density of the thick filament region and the thin filament region, and that the scattering length density of the thick filament changes as resting muscle is put into the rigor state.

2. Materials and methods

2.1. Sample preparations

Bullfrogs (*Rana catesbeiana*) were killed by decapitation, followed by destruction of the spinal cord. Live sartorius muscles were then immediately dissected and used for the neutron diffraction experiments. Muscles at rest length were mounted on the specimen chamber horizontally, slightly stretched in order to keep their fiber axis straight during the measurements. Ringer's solutions containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 3 mM HEPES/NaOH (pH 7.2) were prepared in various D_2O concentrations. Oxygenated Ringer's solution was flowed continuously through the chamber at ~10 °C during the experiments, and muscles were kept in the relaxed state.

Muscles in the rigor state were prepared as follows (Yagi, 1992). Dissected sartorius muscles were incubated in relaxing solution (60 mM K-propionate, 20 mM HEPES/KOH (pH 7.0), 5 mM EGTA, 5 mM Mg-acetate, and 5 mM Na_2-ATP) containing 1 mg/ml saponin for ~3 h, followed by washing with relaxing solution. Skinned muscles were then incubated with relaxing solution containing 1 mM *N*-ethylmaleimide (NEM) to suppress the ability of myosin heads for the development of tension for ~3 h. The solution was then changed into rigor solution (60 mM K-propionate, 20 mM HEPES/KOH (pH 7.0), 5 mM EGTA, and 5 mM Mg-acetate) and incubated for ~1 h. Muscles thus prepared (NEM-rigor muscles) were mounted on the specimen chamber similarly to those in the relaxed state. Rigor solutions containing various D_2O concentrations were flowed continuously through the chamber at ~10 °C during the experiments.

2.2. Neutron diffraction experiments

Continuous flow of Ringer's solution or rigor solution allowed the D_2O concentration within muscle to reach equilibrium with that of the solution. Whether or not the equilibrium was attained was monitored by the transmission of neutrons through the specimen. After ~1 h equilibrium was attained, after which the neutron diffraction measurements were started. Although the equilibrium in the solvent was attained, exchange of labile protons to deuterium (*H–D* exchange) on the protein region might continue. If this continuing *H–D* exchange has a significant effect, it should be detected by the transmission measurements before and after the neutron diffraction measurement because the solution was continuously circulated during the measurement. We measured the transmission before and after each diffraction measurement, but did not detect any systematic changes as a sign of the continuing *H–D* exchange. Thus, in practical terms, the effect of the continuing *H–D* exchange was negligible. We employed average values of the transmission before and after the diffraction measurements as the transmissions for the scale factors (see below). The measurements were done with the small-angle neutron scattering instrument (SANS-J) at the guide hall of the reactor JRR-3M in Japan Atomic Energy Agency, Ibaraki, Japan (Koizumi et al., 2006). A wavelength (λ) of incident neutrons was set to 0.65 nm with a spread ($\Delta\lambda/\lambda$) of 13.8% (the flux was about 1.1×10^5 neutrons/cm^{2}s at a sample position). The beam with a diameter of 8 mm at the sample position was used. The two-dimensional diffraction patterns were recorded with a ^{3}He-2D position sensitive detector, which has a circular sensitivity area with a radius of 290 mm and spatial resolution of 5×5 mm^2, at a specimen-to-detector distance of 3.8 m. With this set-up, measurements at ~10 nm resolution were possible. Exposure time was between 10 h for muscles in D_2O and 36 h for muscles in H_2O. Possible alterations of muscles such as progression of the rigor state during the exposures were monitored by collecting the diffraction patterns in 1 h-time frames. No sign of systematic changes in the diffraction patterns was observed, indicating that no significant structural alterations occurred during the exposures. This was also another sign of the negligible effect of the continuing *H–D* exchange. The diffraction patterns were scaled by the intensity of the incident neutrons, the transmission of the samples, and the exposure time. Because of the rather long exposure times, a complete set of data could not be collected from one specimen. Scaling between different muscles thus had to be done. For each specimen, the diffraction pattern in 100% D_2O was measured in addition to the measurements in other D_2O concentrations. Integrated intensities of strong reflections ((1, 0) and (1, 1) Bragg reflections on the equator, or the 3rd order reflection (M3) due to the thick filaments with an axial periodicity of 42.9 nm or 43.5 nm on the meridian) in the diffraction patterns in 100% D_2O were calculated as below. Ratios of these integrated intensities were then used as scaling factors for different specimens.

2.3. Data reduction

The diffraction patterns were, after finding their origins and correcting their inclination angles, folded across the meridian or the equator, for reduction of the equatorial reflections or the meridional reflections, respectively. The equatorial reflections in the folded patterns were analyzed by projecting the intensity in the meridional direction onto the equatorial axis to produce a pattern of integrated intensity as a function of pixel number. The background of this distribution was determined by connecting the levels at which a bend in the slope was observed on either side of the equatorial