X-ray fiber diffraction of the indirect flight muscle of *Lethocerus indicus*

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Introduction

Glycerinated insect flight muscle (IFM) from the giant waterbug *Lethocerus sp*. is an especially attractive system for structural analysis of myosin crossbridge shapes and actions. Even after permeabilization, glycerination, and months to years of storage at -80°C, its near-crystalline lattices of myofilaments and crossbridges preserve unimpaired mechanical and structural responses in (and transitions between) a multitude of physiological and pharmacological states. When relaxed, the molecular motor heads of four myosin II molecules project perpendicular to the thick filaments as eight-head crowns that are packed into four-fold shelves repeating axially every 14.5 nm [1, 2, 3, 4]. When activated, these heads generate force by ATPase-coupled cyclical interactions with adjacent actin-containing thin filaments. Among all cross-striated muscles, IFM has provided some of the richest x-ray diffraction [5, 6, 7, 8], the clearest thin-section electron microscopy (EM) images of crossbridges and the only three-dimensional EM reconstructions so far of crossbridges *in situ* [3, 9, 10, 11, 12, 13, 14, 15, 16], recently extended to include three-dimensional atomic modeling of crossbridges in quick-frozen contracting IFM [17]. X-ray diffraction arises from the hexagonal filament lattice of IFM (53 nm inter-thick filament spacing) and the helical substructure of its myofilaments with helix repeats ranging from 5 to 39 nm, with pitches ranging from 5 to 160 nm, giving layer lines indexing to n/(232 nm^{-1}). It is notable that in all states, not just rigor, the primary helical repeat of both thin filaments (two-stranded) and thick filaments (four-stranded) is the same, 38.7 nm, a feature that seems favorable for crossbridge synchronization. The low-angle pattern is quite sensitive to the number, structure, and lattice disposition of crossbridge linkages between myosin and actin.

Methods and Materials

Glycerinated IFM preparations were obtained as described [9, 10]. Some experiments (aimed at obtaining high-resolution data) used bundles of 50–80 fibers (L x H x W ~17 x 2 x 0.5 mm) isolated from the muscle. These were oriented in simple Lucite x-ray cells by gluing the muscle ends to stainless steel hooks and stretching fibers until straight as described [7]. For simultaneous force measurements, we mounted smaller bundles (12–25 fibers) in a modified commercial x-ray cell (Güth Muscle Research System, [18]) that incorporated temperature control and a force transducer. Solutions were as described by Tregear *et al*. [8]. Small-angle x-ray diffraction measurements used the Bio-CAT undulator-based beamline at the Advanced Photon Source, Argonne National Labs, Argonne, Illinois. The x-ray diffractometer had sample-to-detector distances 2 or 5 m. X-ray wavelength was 1.03 Å. All flight paths were evacuated except for a small gap around the sample chamber itself (~1 cm downstream, 2 cm upstream of the sample). The beam size at the sample position was about 0.4 x 0.8 mm, and about 0.040 x 0.2 mm (Vertical x Horizontal) at the detector and contained a maximum incident flux of ~1-2 x 10^{13} photons/s. X-ray diffraction patterns were collected with a CCD-based x-ray detector constructed by Walter Phillips' group Brandeis University (1024 x 1024 pixels, 60 mm x 60 mm active area) or with 20 x 25 cm Fuji BAS V image plates that were read out on a BAS200 scanner.

Results

Figure 1 shows a pattern from IFM in the relaxed state taken with the (10X attenutated) equivalent of a 10 ms exposure. The fine focus and low divergence has yielded extremely well-resolved layer lines and diffraction spots. The high signal-to-noise ratio has brought out features not seen before in this state. Most notable of these are the spots on the first row line on the 19.3 nm layer line, which greatly intensify during contraction as myosin heads bind to helically restricted "actin target zones" half-way between the troponins on the thin filament [8]. We are currently modeling the myosin layer lines (labels on right) in these high-quality patterns with a globally exhaustive range of computed three-dimensional models of the relaxed thick filament, building on the initial IFM model of Hudson [4] and following the procedure used by Hudson *et al*. [19] to refine an optimum model (R factor 3% at 6.5 nm resolution) of the relaxed thick filament in bony fish muscle.

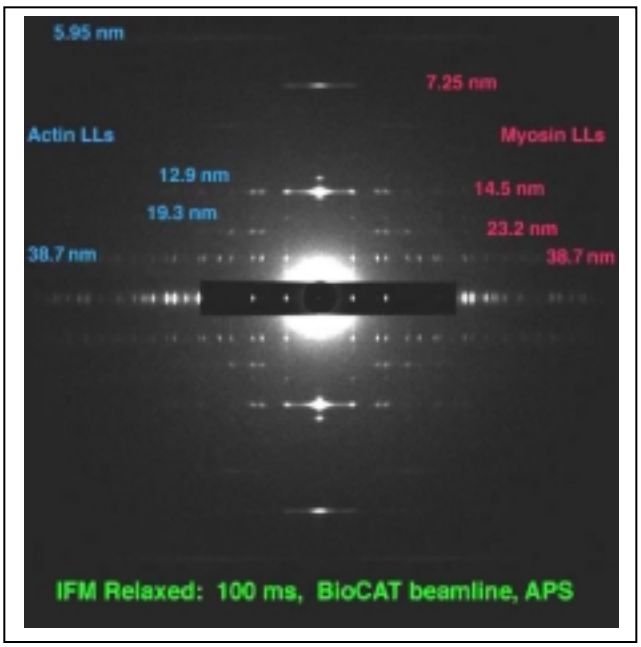

Figure 1: Pattern from IFM in the relaxed state taken with the (10X attenuated) equivalent of a 10 ms exposure.

The native relaxed-state IFM model will provide us with a basis for refined modeling of active and other physiological states, for refinement to fit higher resolution data, and for ultimately extending these models to include thin filaments and all other sarcomere structures.

Figure 2 shows the fine structure in the 14.5 nm reflection that we can observe with the 5 m camera, resolving 12,000 Å order to order splitting. This substructure comes from interference between myosin heads in the two halves of the bipolar myosin filament [20]. The intensity and spacing of the interference bands is a sensitive function of crossbridge tilt and shape in vertebrate muscle Lombardi, 2000 #14309; Huxley, 2000 #14310, so that by coupling x-ray diffraction with ≤1 millisecond mechanical transients that briefly synchronize crossbridges action, one can expect to detect changes in lever-arm tilt of crossbridges. Analysis of these interference features promises even more sensitive measures of crossbridge changes than the 14.5 nm intensities used so effectively of late to identify and distinguish elastic and active changes in conformation [21, 22]. One advantage of the insect system is that its high degree of order encourages direct comparison with electron microscopy, now in progress, of quickly frozen crossbridges synchronized by similar rapid mechanical transients.

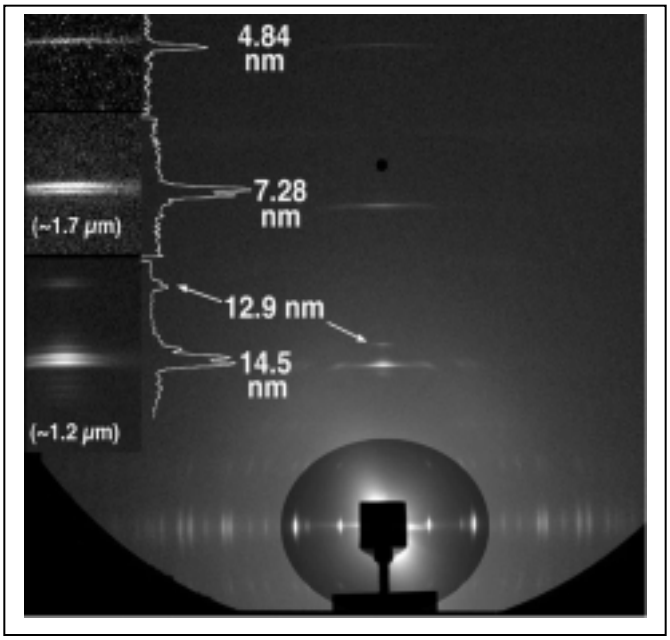

Figure 2: Fine structure in the 14.5 nm reflection.

We must distinguish radiation damage from native causes of intensity change to interpret our physiologically varying patterns. We used decline in peak intensity of the 14.5 nm reflection to measure radiation damage. This declines linearly with dose (Figure 3) at 23°C declining by 4–6% per 10 ms shot. This was unaffected by up to 0–100 mM added dithiothreitol (DTT) which had been previously reported to protect against radiation damage [23]. Cooling to 2ºC showed a substantial improvement, reducing the 14.5 nm intensity loss to 1–2% per 10 ms shot.

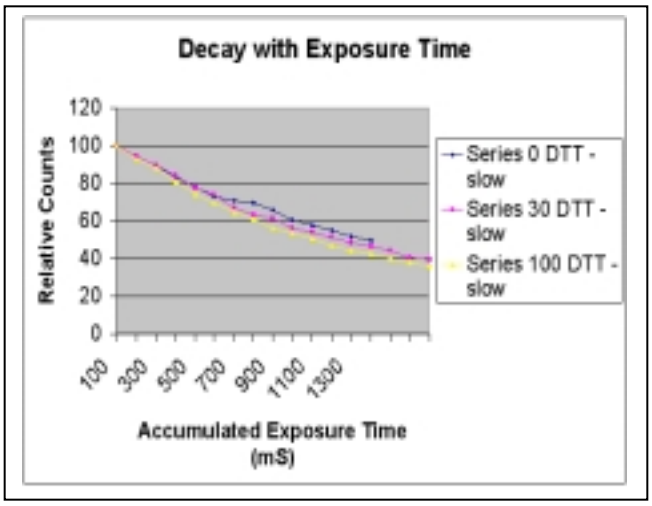

Figure 3: Decline of 14.5 nm intensity with accumulated exposure. No radioprotective benefit was seen from including DTT, nor (not shown) from changing intershot intervals from 5s to 180s.

Discussion

The high quality of the low-angle patterns we have obtained will be very advantageous for our program of modeling filament structures *in situ* in the filament lattice and coordinating with electron microscopy studies. We think it likely that we have now resolved everything there is to resolve in this low-angle region. We can now detect and distinguish with great sensitivity the subtle variations and transitions in the pattern produced by to phenomena such as weak or strong binding of crossbridges, temperature responses (these bugs can only fly after warmup of IFM to 40°C), mechanical transient response to sudden jerks and releases, and the effects of nucleotide and phosphate analogs believed to mimic arrested stages of the crossbridge cycle, etc.

Radiation damage is a serious impediment to native structure and behavior that must be minimized. We have seen that radiation damage can be reduced by a useful amount by lowering the temperature to 2°C, useful for static states but not for exploring active contraction and warm-up responses. Rapid freezing of fibers offers an option that we plan to pursue to study static states, but is again not ideal for uniform trapping of dynamic-state features and cannot be used when simultaneous physiological measurements are required. We must therefore explore the radioprotectant value of soluble agents such as catalase, superoxide dismutase, and even aminothiols [24].

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