EUROPEAN SYNCHROTRON RADIATION FACILITY

INSTALLATION EUROPEENNE DE RAYONNEMENT SYNCHROTRON

Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

Once completed, the report should be submitted electronically to the User Office via the User Portal:

https://wwws.esrf.fr/misapps/SMISWebClient/protected/welcome.do

Reports supporting requests for additional beam time

Reports can be submitted independently of new proposals – it is necessary simply to indicate the number of the report(s) supporting a new proposal on the proposal form.

The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

Reports on experiments relating to long term projects

Proposers awarded beam time for a long term project are required to submit an interim report at the end of each year, irrespective of the number of shifts of beam time they have used.

Published papers

All users must give proper credit to ESRF staff members and proper mention to ESRF facilities which were essential for the results described in any ensuing publication. Further, they are obliged to send to the Joint ESRF/ ILL library the complete reference and the abstract of all papers appearing in print, and resulting from the use of the ESRF.

Should you wish to make more general comments on the experiment, please note them on the User Evaluation Form, and send both the Report and the Evaluation Form to the User Office.

Deadlines for submission of Experimental Reports

- 1st March for experiments carried out up until June of the previous year;
- 1st September for experiments carried out up until January of the same year.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report, in English.
- include the reference number of the proposal to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

ESRF	**Experiment title: The molecular mechanism of regulation of muscle contraction**	**Experiment number**: LS-2514
Beamline:	**Date of experiment**: from: 30.03.2016 to: 03.04.2016	**Date of report**: 16th march 2017
Shifts:	**Local contact(s)**: Theyencheri Narayanan	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

Prof. **Gabriella Piazzesi***, University of Florence, Italy

Prof. **Vincenzo Lombardi***, University of Florence, Italy

Prof. **Marco Linari***, University of Florence, Italy

Prof. **Massimo Reconditi***, University of Florence, Italy

Prof. **Malcolm Irving***, King's College London, UK

Report: Muscle contraction is generally considered in terms of the interaction between two proteins, myosin and actin, polymerized respectively in the thick and thin filaments that overlap in the structural unit of muscle called the sarcomere. However the physiological performance of muscle in vivo, and in particular its regulation and response to external mechanical conditions, is an integrated response of the whole sarcomere, involving proteins other than myosin and actin. In particular, increasing interest focuses on two components of the thick filament: the giant protein **titin**, that runs from the midpoint of each thick filament to its tip, then extends from the thick filament tip in parallel with the thin filaments to the end of the sarcomere, and the **myosin binding protein C** (**MyBP-C**), a thick filament component that can bridge thick and thin filaments in resting muscle. The present experiments were aimed at elucidating the contribution of these two proteins to the recently discovered stress-sensing mechanism that controls the load-dependent recruitment of myosin motors in striated muscle (Linari *et al*. 2015, *Nature* **528**:276-279 ; Reconditi *et al*. 2017, *PNAS*, doi:10.1073/pnas.1619484114), and how their action is modulated by other regulatory factors like the change in overlap between the filaments, the level of intracellular [Ca^{2+}] and the stress on the thick filament. For this, time-resolved X-ray diffraction patterns were collected at ID02 from resting muscle cells (fibres, isolated from the skeletal muscle of the frog), subjected to stepwise rise in the passive force (isotonic stretch protocol), starting from a sarcomere length (SL) of 2.5 μm, which represents the lower limit of full overlap between actin and the MyBP-C-containing region of the thick filament. The X-ray signals analysed in this report are those marking the degree of activation of the thick filament: the first order myosin layer line (ML1), which originates from the resting helical symmetry of myosin motors on the thick filament; the M6 reflection, the spacing of which measures the extension of the thick filament and rises by 1.5% with the development of active isometric force (T_0); the M3 reflection, originating from the 14.34 nm axial repeat of the myosin motors along the thick filament, the intensity and fine structure of which mark the structural transition of motors from the resting state, when they lie on the surface of the thick filament, to the actin-attached force-generating state, characterized by a ~90° tilt toward the perpendicular to the filament axis.

Muscle fibre preparation and protocol. Frogs (*Rana esculenta*), cooled to 2-4 °C, were killed by decapitation followed by destruction of the brain and the spinal cord in accordance with EU official regulations on Use of Laboratory Animals, and of the University of Florence Ethical Committee (in compliance with the rules of the Decreto Legislativo of Italian Government 4 marzo 2014, n. 26). Small fibre bundles (2-3 fibres) were dissected from tibialis anterior muscles, taking care at minimising the length of the tendon attachment at the two ends. The bundles were then transferred to an experimental chamber containing

Ringer's solution at 4°C and mounted vertically at beamline ID02 between a capacitance force transducer and a loudspeaker-coil motor, carried by micromanipulators for adjustment of the bundle length and position relative to the X-ray beam. Two mylar windows were moved as close as possible to the preparation to reduce the X-ray path in water. X-ray patterns were collected on the FReLoN CCD detector with 2048 x 2048 pixels (active area 50x50 mm^2). The relation between fibre length and sarcomere length in the resting fibres was measured by exploiting the beamline capability to perform direct sarcomere length measurements on X-ray patterns collected with a 30 m camera length. For the structural studies, the X-ray patterns were recorded with a camera length of 1.5 m (to collect up to the sixth order of the myosin-based reflections). Radiation damage was minimised by translating the bundle along its axis by 100 μm between X-ray exposures and using fast tandem shutters to limit the exposure time to the acquisition time. Corresponding frames in the time series from subsequent contractions were added to improve S:N. The viability of the fibres was periodically checked by eliciting the isometric contraction with electrical stimulation through two electrodes running parallel to the fibres. The experiment was terminated when the isometric tetanic force T_0 was reduced by more than 15 % below the value at the start of the experiment (~200 kPa).

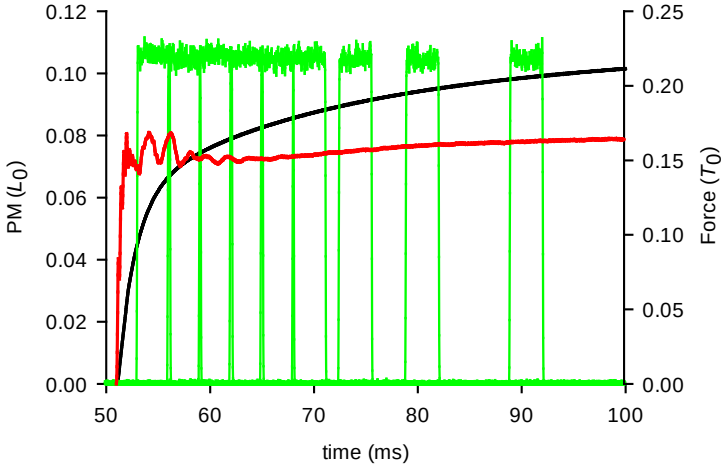

Fig. 1. Experimental protocol: a sequence of 3 ms time frames (green trace, recorded by a pin diode) records the changes in X-ray patterns during the lengthening response (black trace, measured relative to the bundle length L_0, left ordinate) to a stepwise increase in force of 0.17 T_0 (red trace) imposed on a resting muscle at sarcomere length 2.5 μm. The first time frame is set at 2 ms from the start of the force step.

Force steps of ~0.16 T_0 were imposed on the resting fibres (Fig. 1) at the initial length L_0 (corresponding to SL = 2.50 μm). The sarcomere length (SL) increased abruptly to ~2.65 μm during the first 5 ms and then continued to increase more slowly to reach a roughly steady value of 2.75 μm within 100 ms after the force step. X-ray patterns were acquired in 3 ms windows at different times following the step (Fig. 1). X-ray patterns were also collected at SL 2.65 μm and 2.75 μm, attained by slowly lengthening the fibres and thus in the absence of any passive force.

Results. In the frog fibre at rest, due to the relatively stiff tendon attachments and to the absence of the connective tissue that in the whole muscle provides a parallel elasticity, the relation between fibre length and SL is linear in the SL range explored. In fact, the passive force–SL relation obtained with slow lengthening of frog fibres starts to rise just above 2.75 μm. This allows overcoming the complications encountered, in this respect, using whole mammalian muscles (LS 2378). The increase of SL to 2.75 μm, obtained by imposing a slow lengthening, produces: reduction by 20% of the intensity of the first order myosin layer line (I_{ML1}), increase by 0.13% of the spacing of the M6 reflection (S_{M6}), no change in the intensity and fine structure of the M3 reflection (I_{M3}). These results are in agreement with what reported in literature (Reconditi *et al*. 2014, *J. Physiol.* **592**:1119–1137). When SL 2.75 μm is reached with a 0.16 T_0 force step, I_{ML1} reduces by 25%, S_{M6} increases by 0.32% (much more than expected from the thick filament compliance) and I_{M3} does not change. These responses do not show any significant dependence on the time elapsed following the force step.

Conclusions. The results show that in the resting fibre the rise of force to 0.16 T_0 induces structural changes in the thick filament that add to those induced *per se* by the increase in SL, showing that also at rest, when the intracellular $[Ca^{2+}]$ is $<10^{-7}$ M, the thick filament acts as a stress sensor as in the activated fibre (Linari *et al*. 2015).