EUROPEAN SYNCHROTRON RADIATION FACILITY

INSTALLATION EUROPEENNE DE RAYONNEMENT SYNCHROTRON

ESRF

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

Deadlines for submission of Experimental Reports

Experimental reports must be submitted within the period of 3 months after the end of the experiment.

Experiment Report supporting a new proposal ("relevant report")

If you are submitting a proposal for a new project, or to continue a project for which you have previously been allocated beam time, you must submit a report on each of your previous measurement(s):

- even on those carried out close to the proposal submission deadline (it can be a *"preliminary report")*,
- even for experiments whose scientific area is different form the scientific area of the new proposal,
- carried out on CRG beamlines.

You must then register the report(s) as "relevant report(s)" in the new application form for beam time.

Deadlines for submitting a report supporting a new proposal

- 1st March Proposal Round - **5th March**
- 10th September Proposal Round - **13th September**

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.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report in English.
- include the experiment number 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:** Structural basis of regulation of skeletal muscle	**Experiment number**: LS-2721
Beamline:	**Date of experiment**: from:09.02.2018 to: 13.02.2018	**Date of report**: 2nd March 2020
Shifts:	**Local contact(s)**: Theyencheri Narayanan	*Received at ESRF:*

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Report: Introduction. Contraction at the level of the sarcomere, the structural unit of the striated (skeletal and cardiac) muscles is due to the cyclical interactions of myosin motors extending from the thick filaments and the nearby actin containing thin filaments.The aim of this project is to investigate the structural basis of the regulation of the contraction, which involves proteins other than the contractile proteins myosin and actin. In the last five years X-ray diffraction experiments at ID02[1,2,3] have demonstrated that, beyond the classical Ca^{2+}-dependent thin filament activation that makes the actin available for interaction with the myosin motors, a mechanosensing mechanism in the thick filament recruits myosin motors from their resting (OFF) state as a function of the load of the contraction. Increasing interest for explaining the molecular mechanism of thick filament regulation focuses on two sarcomeric proteins, (*i*) the giant protein **titin** which 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. Titin forms an I–band spring that likely provides the load to prevent development of inhomogeneity in serially linked half-sarcomeres with different force capability[4], but may also transmit external stress to the thick filament; (*ii*) the **myosin-binding protein C (MyBP-C)**, which is bound to the backbone of the thick filament in the central one-third of each half (C-zone) and appears to be involved in the control of the OFF state of the motors but also bridges thick and thin filament with its N-terminal[5]. The research is aimed at verifying how Ca^{2+}-dependent thin filament activation signal is transmitted to the thick filaments by MyBP-C putative links and at clarifying the role of titin in both the maintenance of sarcomere length homogeneity and thick filament mechanosensing. For this, we use the intense - highly collimated beam at ID02 to record the low-angle interference X-ray diffraction pattern from intact fibres of frog muscle, reporting the structural changes in the filaments and in the myosin motors. During LS-2721 we have first completed the experiments started with LS-2514, in which rapid changes in force were imposed on the resting fibre to determine the stress sensitivity of the myosin-based reflections in the absence of Ca^{2+}. Then, we have established a new protocol to verify if (*i*) the putative MyBP-C links responsible for the fast communicating path between thin and thick filament and (*ii*) the putative titin ability to couple the load on the thick filament to the OFF-ON switch of motors are Ca^{2+}- sensitive. For this it is ncessary to record the structural changes in the filaments following a stepwise change in force imposed on the stimulated fibre without the confounding effects of the force and stiffness of myosin cross-bridges. During LS-2721 we have tested the effectiveness of the myosin inhibitor blebbistatin[6] in preventing motor attachment and force generation in electrically stimulated fibres and done preliminary measurements on the responses to the force step imposed at various times following the start of stimulation.

Muscle fibre preparation and protocols. 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 bundles of 2-3 fibres were dissected from either lumbricalis or 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 in the X-ray beam path. 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 sarcomere length (SL) in the resting fibres was set to either 2.15 μm (the length for full filament overlap, with the corresponding fibre length indicated as L_0) or 2.7 μm (the length for the development of constant titin stiffness[4], attained exploiting the linear relation between fibre length and SL in an intact frog fibre). The X-ray patterns were recorded with a camera length of 1.6 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 to the acquisition time. The isometric tetanic force at 2.15 μm (T_0) was preliminarily measured to calibrate, for each fibre, the size of the force step (0.25 T_0) imposed either at rest or during tetanic stimulation with the contraction inhibited by the presence of blebbistatin (10 μM). To follow the structural changes accompanying the forcible lengthening response, we collected 5ms frames as various times following the force step. Corresponding 5 ms frames in the time series from each protocol were added to improve S:N.

Results. The X-ray patterns collected following a force step of 0.25 T_0 imposed on the resting fibre show that the spacing of M6 reflection (originating from a periodicity on the thick filament backbone) increases by ca 1.5% per T_0 (that is more than fivefold the increase expected from the instantaneous compliance of the thick filament[7]), suggesting that even at rest the thick filament undergoes stress dependent structural changes. However, all the changes induced by the force step in the other myosin-based reflections marking the OFF state of the myosin motors are not significantly different from those measured in the resting fibre brought to the corresponding SL without force step[8]. As far as blebbistatin effects, one hour after the start of perfusion of solution containing 20 μM blebbistatin, able to fully suppress the development of force under tetanic stimulation, the resting X-ray diffraction pattern does not show changes in either the intensity of the first myosin based layer line or the intensity and fine structure of myosin and actin based meridional reflections reporting the regulatory state of the thick and thin filaments respectively. Moreover, during tetanic stimulation blebbistatin treatment preserves the resting features of the myosin-based reflections.

Conclusions. The results show that, in the resting fibre, the increase in force induces the plastic-like structural extension of the thick filament that, in the activated fibre, accompanies the switching ON of the myosin motors, while the myosin motors remain in the OFF state. Blebbistatin treatment that prevents motor attachment and force generation upon stimulation represents a powerful tool for the planned time-resolved investigation of the Ca^{2+}-dependent structural dynamics of MyBPC-links and titin.

References. 1. Linari *et al*. 2015, *Nature* **528**:276-279. 2. Reconditi *et al.* 2017 *PNAS.***114**:3240-45. 3. Piazzesi *et al*., 2018 *Front. Physiol*. 9:736-743. 4. Powers et al. 2020, *J Physiol.* DOI: 10.1113/JP278713. 5. Luther *et al*. 2011, *PNAS* 108:11423-28. 6. Kovacs et al. 2004, *J Biol Chem* **279**: 35557-63. 7. Reconditi *et al*. 2004, *Nature* **428**:578-81. 8. Reconditi *et al.* 2014 *J Physiol* **592**:1119-37