Practical 2: The major muscle protein actin: assembly and functional assay

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Introduction:

A. Structure of the muscle sarcomere

In vertebrates, three types of muscle can be defined: skeletal muscle, which is responsible for animal movement; smooth muscle, which lines the gut and reproductive tracts; and cardiac (heart) muscle. Skeletal muscle is composed of a bundle of myofibrils which in turn are built from several multinuclear muscle cells. The striation of the myofibrils, when viewed under polarized light, is a consequence of the regular arrangement of thick (Myo) and thin filaments (TF) in the sarcomere, and it appears as a pattern of alternating light and dark bands. Each sarcomere contains a central dark region, called the A-band. The central zone of this region which includes the M-line is mainly composed of myosin containing thick filaments; the central area devoid of actin is referred to as the H-zone. The alpha-actinin containing Z-disc terminates the sarcomere at both ends and anchors the actin-containing thin filaments that extend towards the I-band. Both filament types appear in the flanking region of the A-band.

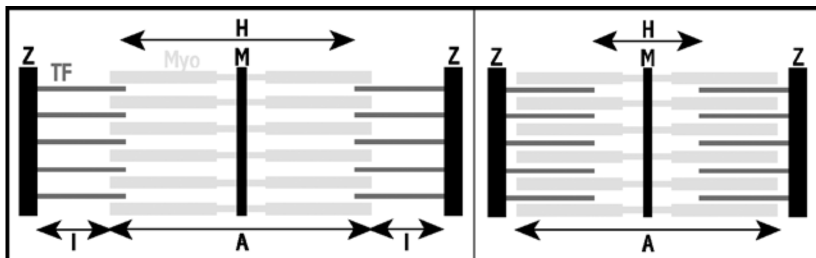


Fig.1. Structure of the sarcomere. On the left is a scheme of the sarcomere in the relaxed state, on the right in the contracted state. TF: actin containing thin filaments (red); Myo: myosin containing thick filaments (cyan).

B. The myosin-containing thick filament

The thick filament is a bipolar rod assembled by the interaction of individual myosin molecules. Each molecule consists of 6 polypeptide chains, with two identical heavy chains and two pairs of light chains (regulatory and essential light chains). They form the 520 kDa holoenzyme that mediates muscle contraction by its ATPase activity. Each heavy chain consists of a globular domain, that carries the ATPase function, and a 134 nm long alpha-helical chain that forms a coiled-coil tail upon parallel dimerization of the 2 heavy chains.

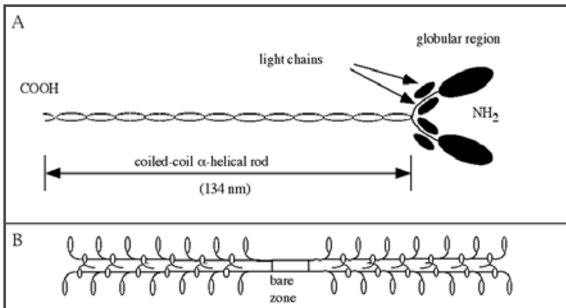


Fig.2. Schematic representation of the myosin molecule. The domain organization of the myosin monomer is shown in (A). A long coiled-coil rod connects to the globular domain by a flexible neck region that carries the light chains. The globular domain has an ATPase function that is the basis for the power stroke during muscle contraction. Upon polymerization of the rod region, the myosin filament shows a bipolar structure with a bare zone in the centre (B).

C. The thin filament

Muscle thin filaments are composed of actin. The regulatory complex consists of tropomyosin with troponin attached to it.

Actin is a globular protein (G-actin) that polymerizes into a filamentous form (F-actin) at increased ionic strength. It is one of the main muscle proteins, but also functions as structural protein for cell arrangement, cellular movement, and the organization of the cytoplasm.

At low ionic strength, actin exists in its monomeric form. Several low affinity binding sites for divalent cations are occupied after an increase in salt concentration. This lowers the net charge of the monomer and results in ATP hydrolysis and polymerization into the filamentous form (F-actin). Muscle contraction is regulated by Ca^{2+} ions that are stored in the sarcoplasmic reticulum and released by nervous stimulation. An increase in the Ca^{2+} concentration activates the myosin ATPase. The regulatory proteins troponin and tropomyosin transmit this effect to the actin-filaments.

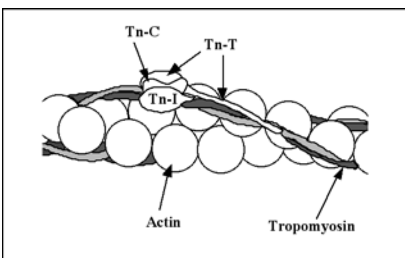


Fig.3. Organization of the vertebrate regulatory components on the actin filament (from Lees-Miller et al., 1991). Troponin I (TnI), troponin C (TnC) and the C-terminal part of troponin T (TnT) binds two-thirds along the tropomyosin (Tm) molecule. The N-terminus is extended towards the C-terminus of Tm.

D. Muscle contraction

Muscle contraction is caused by the cyclic interaction of myosin and actin filaments. Each sarcomere in the muscle fibre shortens symmetrically by about 1/3 of its length during muscle contraction (Fig.1). Thick and thin filaments slide past each other. They are connected by crossbridges from the myosin heads. The power of muscle contraction is produced by the cyclic association and dissociation of these crossbridges between actin and the myosin S1 heads in combination with ATP hydrolysis.

In the rigor state myosin is free of bound nucleotide (Fig.4.A). The tight binding of actin and myosin is weakened by the binding of ATP to the S1 head until it dissociates (Fig.4.B). The ATP hydrolysis into ADP and Pi induces a metastable state with bound products (Fig.4.C). Myosin binds again to actin after several conformational changes (Fig.4.D). The nucleotide binding site has a low affinity for gamma-P in the closed conformation. The release of Pi induces the start of the power stroke and the myosin molecule returns to the relaxed conformation (Fig.4.A).

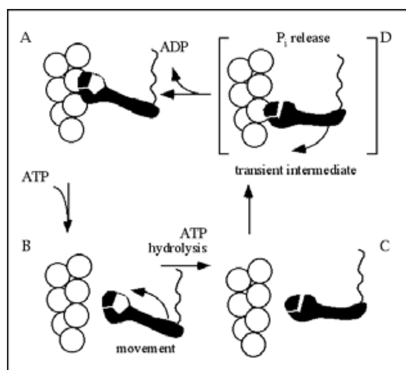


Fig.4. Scheme of the different states of the crossbridge cycle. They differ in the actin-S1-interactions, the nucleotide binding state and their structure. Conformational changes mainly occur in the nucleotide binding pocket and the orientation of the neck region (from Rayment et al.,1993).

Extraction of G-actin, polymerisation into F-actin and viscosity measurement:

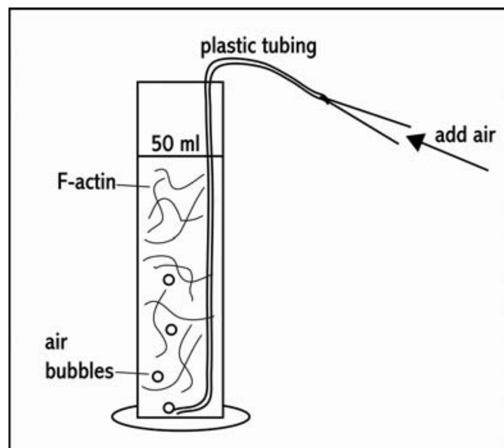
Material:

- Buffer-G — 2mM Imidazole, pH 7.3; 0.2mM ATP; 0.5mM DTT; 0.2mM $MgCl_2$
- 2M KCl
- 1M $MgCl_2$
- 50 ml glass cylinder

- 100 ml glass beaker
- 50 ml falcon tube
- 1.0 ml micropipette
- 0.2 ml micropipette
- 30 cm plastic tubing
- spatula

Procedure:

1. Dissolve 0.5g actin acetone powder in 50 ml buffer-G and stir occasionally for 15 min with the spatula in the glass beaker.
2. Transfer the extract into 50 ml falcon tube and centrifuge in the Heraeus centrifuge for 10 min at 5000 rpm.
3. Decant the supernatant carefully into the 50 ml glass cylinder and add buffer-G to a final volume of 50 ml.
4. Add 1.25 ml 2M KCl dropwise while slowly stirring the solution, then add 0.1 ml 1M $MgCl2$
5. Leave slowly stirring for 15 minutes.
6. Insert one end of the plastic tubing to the bottom of the glass cylinder, attach a 0.2ml pipette tip to the other end.
7. Pipette air into the tube until a small air bubble forms in the solution.



8. Measure the time taken for the air bubble to reach the top of the solution after 2, 5, 10, 15 minutes of polymerisation.

Measurement of ATPase activity of the actomyosin complex:

Material:

- 0.5 ml microtubes
- F-actin from polymerization experiment
- Myosin solution (10 ml) in 0.5 ml microtube
- Quencher solution (Cytoskeleton company, 0.5 ml)
- Activator solution (Cytoskeleton company, 0.5 ml)

Procedure:

1. Add 100 ml of F-actin to 10 ml of myosin in 0.5 ml microtube and incubate at room temperature.
2. Pipette 20 ml of reaction mix at 0, 2, 5 and 10 minutes into new (and labelled) 0.5 ml microtube and place on ice.
3. After all 4 samples have been taken, add 100 ml of quencher and centrifuge for 5 minutes at 14000g at 4°C.
4. Transfer supernatant into new 0.5 ml microtube and add 100 ml of activator and incubate for 10 minutes at room temperature.
5. Transfer 200 ml of the final reaction mix into 96-well microtiter plate and estimate the amount of free phosphate using the phosphate standards.
6. We will measure the optical density of the formed dye at 650 nm wavelength after the practical and provide a copy for you to take home.