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## SESSION I: MECHANOCHEMICAL COUPLING IN MUSCLE I

### Comparison of the energetics of relaxing and fully active muscle fibres from dogfish

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During locomotion dogfish muscle shortens and lengthens alternately in a cyclic way. The muscle is activated and relaxes within each cycle of movement. In many cases the period of stimulation is so brief that the muscle does not become fully active, and for a large part of movement cycle the muscle is relaxing. The energetic properties of fully active muscle are well known. The present experiments were aimed at investigating the energetics of muscle during relaxation and comparing these with the properties of fully active muscle.

In the present study we measured work, heat, and efficiency (work/(heat + work)) during shortening using bundles of white myotomal muscle fibres from dogfish. The muscles were tetanized for 600 ms and shortened for either 286 ms of 143 ms starting at various times after the onset of tetanus. Shortening distance was constant. Alternate tetani were isometric. When the muscle shortened and did work during tetanic stimulation (fully active muscle), its energy output (heat + work) increased above the isometric level (Fenn effect). In contrast, when shortening occurred during relaxation, the muscle did work without an increase in energy cost comparing to the energy output during isometric contraction.

With shortening at a low speed ($3.5\,mm\,s^{-1}$) the efficiency during shortening did not differ between fully active and relaxing muscle. In contrast, at high speed of shortening ($7\,mm\,s^{-1}$) the efficiency during shortening was greater in relaxing muscle than in fully active muscle.

Our conclusion is that the energetic properties of relaxing muscle are different from those of fully active muscle. The difference is dependent on the velocity of shortening.

### ADP release produces a rotation of the neck region of smooth myosin but not skeletal myosin

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Current theories of muscle cross-bridge function suggest that the process of force generation produces a considerable change in the orientation of the neck region of myosin. In order to test this hypothesis, we have studied the orientation of the neck of muscle myosin using electron paramagnetic resonance (EPR) spectroscopy. Recently, electron microscopy has shown that the neck of smooth muscle myosin changes its orientation upon binding of ADP (Whittaker *et al., Nature* **378**, 748, 1995). To further investigate this observation we labelled proteolytic fragments of both skeletal and smooth muscle myosin heads, and diffused them into unlabelled rabbit psoas fibres. A paramagnetic probe was covalently attached to Cys-108 on the light chain-2 (RLC) from chicken gizzard myosin. Labelled RLC was exchanged for native RLC in S1 from both gizzard myosin and skeletal myosin. The EPR spectra indicated a substantial degree of immobilization of the spin probe. The spectra obtained in the absence of ADP for both proteins showed a strong dependence on the orientation of the fibre axis relative to the magnetic field, indicating the probes as well as the myosin neck region were ordered in the rigor state. Modelling the spectra showed that the probes were oriented in a distribution with a full width of 20° for both proteins. Addition of ADP produced a substantial change in the spectrum of probes attached to gizzard S1, but produced only subtle changes in the spectrum of probes on skeletal S1. Spectral simulation showed that the probes attached to gizzard S1 change their orientation by $20° \pm 5°$ upon addition of ADP, while changes for skeletal S1, were less than 5°. The magnitude of the angular change seen for gizzard S1 is in agreement with that observed by electron microscopy. The spectral effect seen for gizzard S1 followed a simple binding isotherm as a function of [ADP] with an apparent affinity constant of approximately 51 M. The spectra of skeletal S1 (± ADP) resembled closely the spectrum of gizzard S1 in the presence of ADP. Thus the rigor gizzard S1 represents a new orientation not seen in skeletal S1. The spectra of spin labelled ADP bound to oriented gizzard muscle were identical to those for skeletal muscle showing that the orientation of the catalytic domains are the same for the two muscles in the presence of ADP. We conclude that both skeletal and gizzard S1 reach the same orientation at the end of their powerstrokes before the release of ADP, and that the neck region of gizzard S1 undergoes an additional rotation upon release of ADP, which does not occur in skeletal muscle. Supported by NIH grant AR42895 (RC).

### Myosin does not contact actin in active muscle so that there is no need for the steric blocking mechanism: it is claimed that regulation is associated with re-structuring of the hydration shells of the proteins

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H. E. Huxley's steric blocking mechanism of muscle regulation, in accord with his (and A. F. Huxley's) swinging-tilting crossbridge theory of muscle contraction takes it for granted that the myosin heads must firmly bind to the thin filaments for tension to develop. The attachment of $Ca^{2-}$ ions to troponin is assumed to cause a shift of the tropomyosin molecules which, in relaxed muscle, presumably inhibit bond formation between myosin and

actin. However, most unfortunately, the basic idea of tight binding in active muscle is, in all probability, absolutely wrong (Oplatka, *Chemical Tracts Biochemistry Molecular Biology* **6**, 18, 1966; *FEBS Letters* **355** 1, 1994) so that myosin head rotation and steric blocking become obsolete. Structural changes in the hydration shells, involving the extrusion of water, intimately coupled with subtle conformational changes, of both 'contractile' and 'regulatory' proteins (including the myosin regulatory light chain), are responsible for both tension development and regulation, which are actually inseparable in living muscle. Support for this picture, just by way of example.

(1) It has been suggested by Geeves and his collaborators that tension development is linked to an isomerization reaction during 'complex' formation between actin and S-1. I have demonstrated that this process involves the release of 'active' water which generates tension and movement. In line with this, Geeves and Halsall (*Biophysics Journal* **52**, 215, 1987) claimed that troponin-tropomyosin inhibits this reaction in the absence of $Ca^{2+}$.

(2) The binding of free $Ca^{2+}$ ions to troponin must be accompanied by the release of part of the strongly bound water of the hydration shells of both $Ca^{2+}$ and troponin. Thus, the reaction is actually $nCa^{2+}$ + troponin → $nCa^{2-}$ − troponin + water. Application of osmotic stress should then shift the equilibrium to the right by removing water and this means increase in $Ca^{2-}$ sensitivity which has, indeed, been observed (see Stephenson & Wendt, *Journal of Muscle Research and Cell Motility* **5**, 243, 1984).

(3) Poo and Hartshorne (*BBRC* **70**, 406, 1976) have shown that activation of the ATPase activity of S-1 by glutaraldehyde-crosslinked F-actin is not controlled by the regulatory proteins. The glutaraldehyde treatment must make actin more hydrophobic, i.e., it should now bind less water, which is what $Ca^{2+}$ does, as seen from the above equation. Apparently, glutaraldehyde makes actin so 'dry' that it cannot anymore participate in the water-releasing and force-generating isomerization reaction. Indeed, such actin does not give rise to superprecipitation (Gadasi *et al.*, *BBA* **333**, 161, 1974). The ATPase cycle must undergo a path which differs from that of native actin, in accordance with Geeves and Hall.

## Increased ATP consumption during shortening in skinned rabbit psoas muscle fibres: effects of $P_i$

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The influence of inorganic phosphate ($P_i$) on the relationship between ATP consumption and mechanical performance under isometric and dynamic conditions was investigated in chemically skinned single fibres or thin bundles from rabbit psoas muscle. Myofibrillar ATPase consumption was measured photometrically by enzymatic coupling of the regeneration of ATP to the oxidation of NADH. NADH absorbance at 340 nm was determined inside a miniature (4 µl) measuring chamber. ATP consumption due to isovelocity shortenings was measured in the range between 0.0625 and 1 $L_0\,s^{-1}$ ($L_0$: fibre length previous to shortening, corresponding to a sarcomere length of 2.64 µm), in solutions without added $P_i$ and with 30 mM $P_i$. To get an estimate of the amount of ATP utilized *during* the shortening phase, quick releases of various amplitudes were performed. After quick releases, sufficiently large so that force dropped to zero, extra ATP was hydrolysed which was largely independent of the amplitude of the release and of the period of unloaded shortening. This extra amount, above the isometric ATP turnover, corresponded to about 0.7 and 0.5 ATP molecules per myosin head at 0 and 30 mM $P_i$, respectively. ATP turnover *during* the isovelocity shortenings was higher than isometric turnover and increased with increasing shortening velocity up to about 2.7 times the isometric value. At low and moderate velocities of shortening (. 0.5 $L_0\,s^{-1}$), $P_i$ reduced ATP turnover during isovelocity shortening and isometric ATP turnover to a similar extent, i.e. a decrease to about 77% between 0 and 30 mM added $P_i$. The extra ATP turnover above the isometric value, resulting from isovelocity shortenings studied at different speeds, was proportional to the power output of the preparation, both in the absence and presence of added [$P_i$]. The effect of shortening velocity and [$P_i$] on energy turnover can be understood in a three-state cross-bridge model, consisting of a detached, a non- or low-force-producing, and a force-producing state. In this model, mass action of $P_i$ influences the equilibrium between the force-producing and the non- or low-force-producing cross-bridges, and shortening enhances cross-bridge detachment from both attached states.

## Mechanical measurements from single frog skeletal muscle myofibrils activated by rapid solution changes

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Single myofibrils isolated from striated muscle represent a viable experimental model for the study of the mechanics of muscle contraction (Bartoo *et al.*, *J. Muscle Res. Cell Motil.* **14**, 498–510, 1993; Friedman & Goldman, *Biophys. J.* **64**, 345a, 1993). With this preparation of extremely small cross-sectional area it is possible to obtain a rapid diffusional equilibrium following changes of the bathing medium. In this study we present a novel experimental method which allows solution changes within 50 ms resulting in rapid increase in calcium concentration in the close environment of the specimen and/or in application of rapid perturbation of the composition of the experimental solution. Experiments were performed using single myofibrils or thin bundles of 2–3 myofibrils, 50–100 µm long, prepared by homogenization of frog glycerinated tibialis anterior muscle. The preparations were mounted horizontally between the lever arms of an isometric force transducer and a length control motor (Colomo *et al.*, *J. Physiol.* **475**, 347–50, 1994) in a temperature controlled trough filled with relaxing solution (pCa 8, 3 mM MgATP; 15°C). Sarcomere length was set just above slack length (2.1–2.2 µm). Mounted myofibrils were continuously perfused by one of two parallel streams of solution jetted by gravity from a theta style glass capillary positioned at right angle with the experimental preparation and at a distance of 500 µm. Each pipette channel (200 µm tip diameter) was connected to reservoirs filled with either relaxing or activating (pCa 4.75) solution. The perfusion system was firmly attached to a stepping motor that could be operated to cause rapid alternation of the stream flowed over the myofibril. The mechanical artefact associated with the movement of the perfusion pipette lasted 100–150 ms. When myofibrils were subjected to cycles of activation and relaxation using this experimental device and at high ATP concentration (3 mM), both tension rise and tension relaxation were complete before the end of the mechanical artefact. The maximal activated force of single myofibrils averaged $0.45 \pm 0.03$ µN ($n = 16$) which corresponds to a mean tension of $376 \pm 21$ kN m$^{-2}$. Myofibrils activated at low MgATP concentration (60 µM) showed a markedly slower time course of tension development. Preliminary results on single myofibrils activated and then subjected to a rapid decrease in MgATP concentration indicate a moderate increase in the level of isometric force developed at the contracture plateau, in agreement

with previous results on whole skinned fibres (Ferenczi *et al.*, *J. Physiol.* **350**, 519–43, 1984).

## The time course of phosphate release at the onset of rabbit muscle contractions, and during length perturbations

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We used the phosphate binding protein assay (Ferenczi *et al.*, *Biophys. J.* **68**, 191–3s, 1995) to monitor the rate of phosphate release in permeabilized muscle fibres of rabbit skeletal muscle to measure the ATPase rate at the beginning of contractions initiated by the photolytic release of ATP from caged-ATP. We also monitor the effect of length perturbations on the rate of phosphate release. The sensitivity and time resolution of the assay allows the observation of changes in phosphate release immediately following the length perturbations, thus providing a new method for investigating the relationship between the hydrolysis of ATP and the elementary steps in the force generation process.

## Strain dependent cross-bridge binding rates related to axial and non-axial distortion of cross-bridges

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Stretch and release experiments with small length changes on skinned fibres of the frog have revealed an increase of the Young's modulus as a function of frequency in the relaxed as well as in the rigor state over a range of 100 Hz up to 50 kHz (De Winkel *et al.*, *J. Muscle Res. Cell Motil.* **14**, 302, 1993). The Young's modulus increases over this range with a power of frequency between 0.1 and 0.5. These data point to state transitions of the cross-bridges with a great variation in reaction rates. This variation is explained in terms of the different distortions of the cross-bridges attached to the actin filament. Importantly, extension of the cross-bridges falls short in explaining the observed variation when only the axial direction is considered. Therefore a concept is developed for cross-bridge attachment that incorporates extension in the axial direction as well as a distortion in the cross-sectional plane of the fibre, caused by the helical structure of the actin filament. From this concept a model is derived for attachment of weakly bound cross-bridges, which enables simulation of the frequency dependence of the Young's modulus for ionic strengths from 30 to 280 mM and for osmotic compression by dextran (mw . 200 000). The concept presented here also has the potential to explain the frequency dependence of the Young's modulus in rigor.

## Kinetics of ATP cleavage and phosphate release steps from associated rabbit actomyosin using a novel fluorescent phosphate probe

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Muscle contraction is thought to occur as the result of a cyclic association and dissociation of cross-bridges formed between myosin molecules in the thick filaments and f-actin molecules in the thin filaments, involving concomitant hydrolysis of ATP. This cycle can lead to relative sliding of the actin and myosin filaments and result in the production of work. A rationale for studying the kinetic mechanism of ATP hydrolysis by actomyosin-S1 in solution is that it may directly relate to the observed physiological and pathological properties of muscle. In the absence of actin hydrolysis is rapid and phosphate dissociation limits the steady state rate. In spite of over 20 years of intensive study the rate constants of the hydrolysis and phosphate release steps in the presence of actin have not been measured nor has it been possible to determine the rate limiting step of the reaction in the presence of actin.

We have measured the kinetics of phosphate (Pi) release during a single turnover of actomyosin (AM) nucleoside triphosphate (NTP) hydrolysis using double mixing stopped-flow fluorescence at very low ionic strength to prevent dissociation of the actomyosin. Myosin-S1 and NTP are mixed and incubated for ~1 s to allow NTP to bind to myosin and generate a steady-state mixture of M-NTP and M-NDP-Pi. The steady state intermediates are then mixed with actin. The kinetics of Pi release are measured using a fluorescent probe for Pi, based on a phosphate binding protein (Brune *et al.*, *Biochemistry* **33**, 8262, 1994). The kinetics of Pi release are biphasic. At saturating [actin], there is a correlation between the amplitude of the fast phase and the size of the Pi burst in the absence of actin: the size of this phase corresponds to the M-NDP-Pi formed during the first mix and the kinetics of the phase is Pi release from AM-NDP-Pi. The slow phase corresponds to the amount of M-NTP present after the initial mix and measures the rate of the cleavage step on associated actomyosin. For ATP at 20§C the rate of the Pi release step for rabbit skeletal myosin-S1A1 is $75 \pm 5\ s^{-1}$, 25 times larger than the cleavage step, which is the rate limiting step of actomyosin ATP hydrolysis at saturating actin. The rate constant of the slow phase of the Pi release (measuring cleavage) is dependent upon the structure of the NTP substrate (CTP ~mantCTP . ATP . mantATP . azaATP . GTP). The rate constant of the rapid phase of Pi release has a very small dependence upon substrate structure but is significantly dependent upon the myosin type (fast skeletal . cardiac . smooth muscle myosin). This work was supported by MRC, UK, NIH AR40964 and the American Heart Association (Virginia Affiliate).

## Diaphragmatic contractile dysfunction in endotoxemic rats is induced by nitric oxide production

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Sepsis is an common cause of morbidity and mortality, particularly in the elderly and/or immunocompromised patients. Respiratory failure is a major clinical manifestation of sepsis, greatly contributing to the mortality of this pathologic condition. Respiratory failure in this context has been traditionally related to the development of the adult respiratory distress syndrome (ARDS). Nitric oxide (NO), a free radical which is cytotoxic and negatively inotropic in the heart and skeletal muscle, is produced in large amounts during sepsis by a NO synthase inducible (iNOS) by LPS and/or cytokines. The aim of this study was to investigate whether iNOS was induced in the rat diaphragm after *E. Coli* LPS inoculation, whether iNOS activity was associated with diaphragmatic contractile dysfunction, and whether inhibition of iNOS