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SESSION I: STRUCTURE OF ACTIN AND MYOSIN

Complexes of truncated myosin head from *Dictyostelium discoideum* in the presence of nucleotide analogs

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The myosin protein is involved in numerous essential cellular processes all relating to the transduction of chemical energy into motion. In all instances, the myosin molecule generates force against an actin filament coincident with the hydrolysis of a single ATP molecule. We have recently solved the structure of the truncated myosin head from *Dictyostelium discoideum* in the presence of a number of nucleotide analogues. The structure of the *Dictyostelium* myosin is very similar to that determined previously for the chicken myosin. Noteworthy structural features are that the asymmetric molecule contains a central 7-stranded β-sheet above which the nucleotide binds. A large cleft splits the 50 kDa region of the protein; the apex of this cleft is very close to the nucleotide binding pocket. A long α-helix at the C-terminus of the truncated head continues toward the light chain binding domain of the heavy chain and on to the myosin II tail.

The S1Dc protein adopts one of two conformations that is determined by the nucleotide present at the active site. Several nucleotide complexes, including Mg·ADP·BeF_x, Mg·PP_i, Mg·AMPPNP and Mg·ATPγS, model the prehydrolysis state of myosin in the presence of Mg·ATP. These complexes define the active site residues of the enzyme. A second class of nucleotide complexes exists which models the transition state for nucleotide hydrolysis. The two compounds in this class are Mg·ADP·AlF_4 and Mg·ADP·VO_4. Several large conformational changes are observed between the two states. In comparison with the prehydrolysis state of the enzyme, the transition state complexes demonstrate a rotation of the lower domain of the 50 kDa region of the protein that results in a closing of the large cleft in this region of the protein. Analysis of the structures of nonhydrolyzed analogues ATPγS and AMPPNP provide clues to the structural basis of the biochemical and biophysical effects of these compounds. The hydrogen-bonding network surrounding the γ-phosphate pocket of the ATPγS structure is unfavourable for the conformation required for nucleotide hydrolysis. A rearrangement of the water structure in the active site surrounding Asn 233 provides an explanation for the decreased affinity of myosin for AMPPNP.

Kinetic characterization of a *Dictyostelium* myosin head fragment with truncated 50/20K junction

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The loop 2 region of myosin (50/20K junction) plays an important role in determining the rate at which ATP hydrolysis is simulated upon binding to actin (Uyeda *et al.*, 1994, *Nature* **368**, 567).

To investigate further the role of loop 2 in myosin function, we constructed a mutant myosin head fragment (NL-M765) that lacks part of the 50/20K junction. Milligram quantities of this mutant protein and the wild-type construct M765 were produced. The enzymatic behaviour of both constructs was compared. Actin binding was studied by co-sedimentation and stopped-flow analysis. Co-sedimentation experiments demonstrated that NL-M765 binds to actin, although with at least 10-fold lower affinity than M765. ATP induces complete release of both M765 and NL-M765 from actin. The quenching of pyrene-actin fluorescence, which occurs upon binding of myosin, was used to characterize the interaction of actin with mutant and wild-type myosin constructs in a stopped-flow set-up. The results obtained with M765 were very similar to those obtained with the wild-type constructs M761, M781 and M864 (Kurzawa *et al.*, 1997, *Biochemistry* **36**, 317), while no quenching of the fluorescent pyrene signal was detected with NL-M765. We also measured the steady-state ATPase activity for both constructs. Basal ATPase activity for NL-M765 was significantly increased when compared with M765 ($0.19\ s^{-1}$ and $0.06\ s^{-1}$ respectively). But stimulation of ATPase activity by actin was strongly decreased for NL-M765. M765 showed a 9-fold stimulation of ATPase activity following the addition of 20 μM actin, while addition of 20 μM actin to NL-M765 gave only a 1.5-fold stimulation of ATPase activity. These results emphasize the role of loop 2 in high affinity actin binding and the coupling between the actin- and nucleotide-binding sites of myosin.

Introduction of charge into myosin loop 2 affects actin-binding and ATPase

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Recombinant *Dictyostelium discoideum* myosin head fragments were used for the examination of actomyosin interaction in solution. The segment between myosin residues 618 and 622 (loop 2) was swapped with sequences enlarged either by the introduction of several neutral GNN- or positively charged GKK-motifs. Mutant constructs with loops carrying up to 20 additional amino acids and charge variations from −1 to +12, in comparison with native *Dictyostelium* myosin, were produced. High synthesis levels were obtained with each construct; between 0.5 and 3 mg of homogeneous, functional protein per g of cells was obtained after purification. Steady-state and transient kinetics were used to characterize the enzymatic behaviour of the mutant motor domains. Eight out of the nine mutant constructs that were characterized showed a 2 to 3-fold increase in basal ATPase activity. In regard to their interaction with actin, mutants with moderate charge changes (−1 to +2) displayed wildtype-like behaviour. The introduction of more than one positively charged GKK-motif resulted in a pronounced effect on the actin-activated

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IANBD-labelled troponin-I reflects changes in thin filament activation, all of our observations are fully accounted for by our previously proposed kinetic scheme. In this concept it is assumed that (i) actin filaments exist in (at least) two different states, an active and an inactive state which are in rapid equilibrium; (ii) this equilibrium is Ca^{2+}-controlled, with increasing occupancy of the active state at high Ca^{2+}; and (iii) phosphate release only occurs when the myosin head is bound to the active form of the thin filament.

Thin filament activation kinetics control the rate of tension redevelopment (k_{tr}) during sub-maximal Ca^{2+} activated contractions in skinned rabbit psoas muscle at 10° C

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The rate of cross-bridge tension production is highly Ca^{2+}-sensitive in isometrically contracting skeletal muscle. To examine if thin filament (TF) Ca^{2+} binding kinetics influences the rate of tension production, we have independently altered the kinetics of thin filament activation or cross-bridge cycling. To alter TF Ca^{2+} binding kinetics, the Ca^{2+} dissociaton rate from troponin C (TnC) was reduced by calmidazolium (CDZ). Alternatively, the rate of cross-bridge cycling could be either increased or decreased (~30%) by replacement of 5 mM ATP with 5 mM 2-deoxy-ATP (dATP) or 0.5 mM ATP, respectively. In maximally Ca^{2+} activated skinned fibres, changes in substrate conditions caused concomitant changes in the rate of tension redevelopment (k_{tr}; Brenner & Eisenberg (1986) *PNAS* **83**, 3452), with little or no effect on isometric tension, while 10 μM CDZ had no effect on either isometric tension of k_{tr}. These results confirm that the rate of tension production in maximally Ca^{2+} activated fibres is dependent on the rate of cross-bridge cycling (Metzger & Moss (1990) *Science* **247**, 1088). Conversely, 10 μM CDZ increases k_{tr} during submaximal Ca^{2+} activations compared with isometric tension-matched control measurements. The following lines of evidence suggest that this effect is not due to an increased rate of cross-bridge cycling, but instead results from a change in TF activation kinetics: (1) in tension-matched submaximal Ca^{2+} activations the velocity of unloaded shortening is increased by dATP but k_{tr} is not increased (Regnier *et al.* (1997) *Biophys. J.* **72**, A379); (2) CDZ does not alter solution ATPase or *in vitro* motility of F-actin; and (3) the extraction of CDZ-exposed TnC and replacement with purified native TnC reverses the effects of CDZ (Regnier *et al.* (1996) *Biophys. J.* **71**, 2786). Additionally, k_{tr} is elevated slightly with 0.5 mM ATP at low levels of Ca^{2+} activated tension, suggesting that strongly bound cross-bridges are activating the TF under these conditions. Taken together, these results suggest that TF Ca^{2+} binding kinetics can control the rate of cross-bridge tension production in sub-maximally Ca^{2+} activated fibres, while the rate of tension production in maximally activated fibres is set by the rate of cross-bridge cycling.

Calcium dependence of the apparent rate of force generation in single frog and rabbit skeletal muscle myofibrils activated by rapid solution changes

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Single myofibrils or small bundles of 2–3 myofibrils 50–100 μm long prepared from glycerinated frog tibialis anterior muscle or from rabbit psoas muscle (Colomo *et al.* (1997) *J. Physiol* **500.2**, 535) were activated using a novel method which allows solution changes within 10 ms. The preparations were mounted horizontally between the lever arms of an isometric force transducer and a length control motor (Colombo *et al.* (1994) *J. Physiol.* **475**, 347) in a temperature-controlled trough filled with relaxing solution (pCa 8, 15° C). Sarcomere length was set just above slack length. Mounted myofibrils were continuously perfused by one of two parallel streams of solution jetted by gravity from a theta-style glass pipette. Each pipette channel was connected to reservoirs filled with either relaxing or activating solutions of different calcium concentration (MgATP 3 mM plus CP/CPK regenerating system). The perfusion system was firmly attached to a stepping motor for rapid alternation of the streams over the myofibrils. When frog or rabbit skeletal muscle myofibrils were activated, tension rapidly rose to steady values that were strongly dependent on calcium concentration. In both cases, the force/pCa relations obtained were consistent with those reported for larger preparations. With all the myofibrils tested, the time course of force development was approximately exponential and superimposable on the time course of force redevelopment following a release–restretch manoeuvre applied at the contraction plateau (Brenner (1988) *Proc. Natl Acad. Sci.* **85**, 3265). At saturating pCa (4.75), the apparent rate constant of the process leading to force generation was 15–20 s^{-1} for the frog myofibrils and half that for the rabbit myofibrils, independently of the experimental method used. These values are in reasonable agreement with those for single skinned fibres from the same muscles of the frog and the rabbit following their activation by the photolysis of caged calcium. In both preparations, the rate constant of force generation was slowed down by decreasing calcium concentration, but the effect was much larger for rabbit than for frog skeletal myofibrils, where it could be detected only at very low levels of activation. These results do not support the idea that the effect of calcium on the kinetics of force generation is larger in fast than in slow muscles (Metzger & Moss (1990) *Science* **247**, 1088; Campbell (1997) *Biophys. J.* **72**, 254).

The pCa/tension relationship is symmetrical about the midpoint

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The slope, n_H, of the pCa/tension data from rabbit psoas skinned fibres when fit to the Hill equation is 5 or greater, which probably reflects extended cooperativity between troponin–tropomyosin regulatory complexes making up the regulatory strands of the thin filaments (Brandt *et al.* (1984) *J. Mol. Biol.* **180**, 379). When the high slope was first described (Brandt *et al.* (1980) *Proc. Natl Acad. Sci. USA* **77**, 4717) the pCa/tension data appeared to be asymmetrical in that the slope of the lower half of the curve was steeper than the upper. Moss *et al.* (1985, *J. Gen. Physiol.* **86**, 585) fit the two halves separately and proposed that the lower, steeper part reflected cooperativity between regulatory complexes, and the upper part cooperatively between Ca^{2+} binding to a single TnC. Here we report that when measures are taken to keep the distribution of sarcomere lengths uniform, all the pCa/tension data lie directly on a symmetrical curve drawn from the Hill equation; the lower and upper points fit the same slope. After dissection, small aluminium clips are gently squeezed on to the fibre segment ends and it is mounted in the apparatus in relaxing solution. The solution is changed by stepping through a series of solution drops between plates that move linearly to envelope the stationary fibre segment attached to the motor and force transducer. The segment remains in the optical path throughout solution change. First it is moved into rigor solution, then momentarily moved into air while small drops of 5% glutaraldehyde in rigor solution (Dantzig *et al.* (1991) *Biophys. J.* **59**, 36a; Linari *et al.* (1993) *J. Physiol.* **473**, 8P) then 10% shellac in 95% ethanol are applied to its ends. After relaxation in pCa 8 buffer, the fibres are exposed to a pre-activation solution, then one test pCa in a closely spaced incremental series, and the tension and sarcomere lengths monitored. The data for each fibre are fit to the Hill equation; for five fibres the mean (SEM) pK is 5.88 (0.05), the n_H is 6.3 (0.7). The mean n_H is higher than previously reported because in all these curves the upper high Ca^{2+} half is as steep as the lower half. The symmetry of the curve rules out the explanation for cooperativity