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MECHANICAL APPROACHES TO THE ELUCIDATION OF THE CROSSBRIDGE CYCLE

Correlation between the crossbridge cycle in muscle and the actomyosin ATPase cycle in solution

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Muscle contraction is believed to be driven by projections from the myosin filaments, the crossbridges, which interact cyclically with the actin filaments, involving the splitting of one ATP molecule per cycle. Various approaches have been used to study this cyclic interaction. In biochemistry, actin and the soluble fragments of myosin, i.e. myosin subfragment-1 (S1) or heavy meromyosin (HMM), are used to define the various states and the kinetics of this cyclic interaction in solution. In physiology, an attempt is made to determine the mechanical characteristics of the various crossbridge states and the kinetics of the crossbridge cycle in the assembled contractile system by using mechanical parameters like force, speed of shortening, or fibre stiffness. A key difference between the biochemical system and the assembled contractile system used in physiology is the fact that changes in the crossbridge configuration are restrained and directed by the filament lattice, a factor which eventually leads to the generation of isometric force or directed shortening. Such deformation or strain of the crossbridges will affect at least part of the kinetics of the crossbridge cycle. Such effects, however, cannot be observed in solution. For this reason experiments were performed to test whether and to what extent some of the characteristics of the actomyosin ATPase cycle, defined by biochemistry, also apply to the crossbridge cycle *in vivo*.

The kinetic schemes of the actomyosin ATPase cycle in solution

One possible scheme of the actomyosin ATPase system which can explain steady state and presteady state data of the biochemical experiments is shown in Fig. 1 [Stein *et al.*, *Biochemistry* **18**, 3895–909 (1979); *Biochemistry* **23**, 1555–63 (1984)]. Other schemes have been discussed, four-state models, i.e. schemes with only four states with ATP or the hydrolysis products bound to the myosin heads [Stein *et al.*, 1979; Webb & Trentham, *J. biol. Chem.* **256**, 10910–16 (1981); Rosenfeld & Taylor, *J. biol. Chem.* **259**, 11908–19 (1984)]. Despite the continuing discussion about the necessary number of states with ATP or the hydrolysis products bound to the myosin heads, all the proposed kinetic schemes have some common features; first, two groups of states are found, one group with weak affinity to actin, the weak-binding states, and one group with strong affinity to actin, the strong-binding states. During each cycle, S1 or HMM is believed to cycle between the weak-binding states and the strong-binding states. Second, all the kinetic schemes have the rate-limiting step located either within the group of weak-binding states (Stein *et al.*, 1979, 1984; Rosenfeld & Taylor, 1984) or representing the transition from the weak-binding states to the strong-binding states (Webb & Trentham, 1981). Independent of the exact location, the rate-limiting step will determine the rate of the transition from the weak-binding states to the strong-binding states when the system is cycling. Third, a major mechan-

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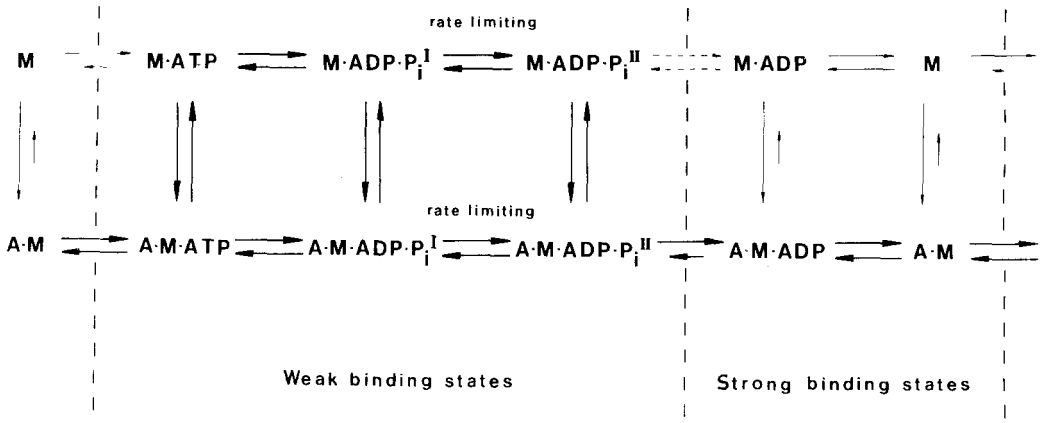

Fig. 1. Kinetic scheme of the actomyosin ATPase cycle (Stein *et al.*, 1979, 1984). This 6-state model includes six states with ATP or the hydrolysis products bound. The predominant pathway for the actomyosin ATPase is indicated by the heavy solid arrows. The dashed arrows indicate the rate-limiting step in the absence of actin. The relative length of the forward and reverse arrows qualitatively indicate the change in free energy across the corresponding step. A, actin; M, myosin subfragment-1.

ism of regulation appears to be the blocking of the transition from the weak-binding states to the strong-binding states since, in the presence of MgATP but without Ca^{2+}, S1 and HMM still bind to actin but do not show significant ATPase activity [Chalovich *et al.*, *J. biol. Chem.* **256**, 575–8 (1981); Wagner & Giniger, *J. biol. Chem.* **256**, 12647–50 (1981); Chalovich & Eisenberg, *J. biol. Chem.* **257**, 2432–7 (1982); Wagner, *Biochemistry* **23**, 5950–6 (1984)].

Weak-binding states and strong-binding states in muscle; some of their characteristics determined by mechanical and X-ray diffraction experiments

To see whether and to what extent these common features of the actomyosin ATPase schemes can also be found in the assembled contractile system, we first addressed the question whether *in vivo* we can find evidence for crossbridge attachment in states equivalent to the weak-binding states of the actomyosin ATPase cycle. From the *in vitro* experiments, a pure population of crossbridges in the weak-binding states should be found in relaxed fibres when the transition from the weak-binding states into the strong-binding states is blocked while binding is still observed. That crossbridges in relaxed fibres might represent the equivalent of the weak-binding states was suggested by the work of Marston [Marston & Tregear, *Nature, New Biol.* **235**, 23–4 (1972); Marston, *Biochim. biophys. Acta* **305**, 397–412 (1973)] showing that in relaxed muscle fibres crossbridges have either ATP or the hydrolysis products bound. In case of analogy between muscle fibres and solution, one should therefore be able to detect crossbridge attachment in relaxed muscle fibres unless actin affinity is too weak for significant crossbridge attachment to occur. To optimize conditions for possible crossbridge attachment in relaxed fibres, we worked at low ionic strength (20 mM) since *in vitro* the actin affinity increases when ionic strength is lowered. Crossbridge attachment was probed for with measurements of apparent fibre stiffness and equatorial X-ray diffraction patterns, using single skinned rabbit psoas fibres. Apparent fibre stiffness was found to be up to 1/2 to 2/3 of the stiffness observed in a fully Ca^{2+} activated fibre [Brenner *et al.*, *Proc. natn. Acad. Sci. U.S.A.* **79**, 7288–91 (1982)]. Furthermore, the apparent fibre stiffness was very closely proportional to filament overlap (Brenner *et al.*, 1982). These data suggest that, at least at low ionic strengths, a significant number of crossbridges is attached to the actin filaments without producing net axial force. This predicts that the mass associated with the actin filaments under these conditions should be significantly larger than the mass of the thin filaments alone. Equatorial X-ray diffraction patterns were therefore recorded from the same single skinned rabbit psoas fibres under relaxing conditions and at the same low ionic strength. The reconstructed two-dimensional electron density maps indeed showed much more mass associated with the actin filaments than expected from the thin filaments alone, approximately as much mass as observed in fully Ca^{2+} activated fibres [Brenner *et al.*, *Biophys. J.* **46**, 299–306 (1984)]. Thus, both stiffness and X-ray

diffraction data are consistent with significant crossbridge attachment in relaxed fibres at low ionic strength, analogous to the binding of S1 or HMM to actin under relaxing conditions in solution.

Another characteristic feature of the weak-binding states found *in vitro* is the rapid equilibrium between association and dissociation [White & Taylor, *Biochemistry*, **15**, 5818–26 (1976); Stein *et al.*, 1979]. Therefore, we searched for evidence as to whether the crossbridges, attached in relaxed fibres at low ionic strength, are also characterized by a rapid equilibrium between attachment and detachment. Crossbridges which detach rapidly might do so during a stretch applied to the fibre to measure fibre stiffness, resulting in a loss of force detected during the stretch. Thus, the slope when force is plotted against change in sarcomere length during the stretch, i.e. apparent fibre stiffness, should decrease with increasing speed of crossbridge detachment and decreasing speed of the applied stretch. We therefore measured apparent fibre stiffness as a function of the speed of stretch [Brenner *et al.*, 1982; Schoenberg *et al.*, In *Contractile Mechanisms in Muscle* (edited by POLLACK, G. H. and SUGI, H.). New York: Plenum Press (1984); Brenner *et al.*, in preparation]. In rigor fibres where the probability of crossbridge detachment during a stretch is small, apparent fibre stiffness changes by only about 15% when speed of stretch is varied over approximately six decades of speeds, up to about 5×10^4 (nm half-sarcomere^{-1}) s^{-1}. In relaxed fibres at low ionic strength, however, a very significant speed dependence was found over this range of speeds. With the fastest stretches, approximately 5×10^4 (nm half-sarcomere^{-1}) s^{-1}, apparent stiffness is about 1/2 to 2/3 of the active stiffness, while at speeds around 10^2 (nm half-sarcomere^{-1}) s^{-1} apparent fibre stiffness is only about 10% of the stiffness observed at the high speed of stretch. Since a significant fraction of crossbridges is attached in relaxed fibres at low ionic strengths, i.e. the binding constant is around unity, the rate constants of attachment and detachment have to be of the same order of magnitude. To get an idea about the rate constants of detachment and reattachment from these measurements one can determine the time necessary for filament sliding over a range over which a crossbridge can remain attached (approximately 10 nm). For our fastest stretches, this time would be about 2×10^{-4} s. Since crossbridges apparently detach and reattach even during our fastest stretches, attachment and detachment have to occur during this time period, i.e. with rate constants up to at least 10^4 s^{-1}. Thus, our mechanical experiments indicate that in muscle as in solution, the weak-binding states are characterized by a rapid equilibrium between attachment and detachment.

A third characteristic quality of the weak-binding states *in vitro* is the decrease in actin affinity with increasing ionic strength [Greene *et al.*, *Biochemistry* **22**, 530–5 (1983)]. We therefore measured apparent fibre stiffness and equatorial X-ray diffraction patterns of relaxed fibres at various ionic strengths. Since apparent fibre stiffness was found to be sensitive to the speed of stretch including the fastest stretches it was possible to apply, the observed decrease in apparent fibre stiffness when ionic strength is increased could at least partly be caused by increased rate constants of attachment and detachment rather than less crossbridge attachment (Brenner *et al.*, in preparation). However, equatorial X-ray diffraction experiments showed a decrease in mass associated with the actin filaments when ionic strength is increased (Brenner *et al.*, 1984). This suggests fewer crossbridges attached at higher ionic strengths.

Thus, stiffness and equatorial X-ray diffraction data strongly suggest that crossbridges in relaxed fibres have similar qualities as described for the weak-binding states of the *in vitro* actomyosin ATPase. To see whether an equivalent of strong-binding states with higher actin affinity and a slower equilibrium between attachment and detachment could be detected [Lymn & Taylor, *Biochemistry* **10**, 4617–24 (1971); Greene & Eisenberg, *J. biol. Chem.* **255**, 543–8 (1980); Marston, *Biochem. J.* **230**, 453–60 (1982)], we studied apparent fibre stiffness (Brenner *et al.*, in preparation) and equatorial X-ray diffraction patterns [Brenner *et al.*, *Biophys. J.* **41**, 33a (1983)] in the presence of $MgPP_i$. This condition is believed to represent an analogue of the strong-binding states. Thus, a pure population of strong-binding crossbridge states is obtainable while in active muscle, i.e. during cycling, both weak and strong-binding states are expected to coexist. Both approaches, stiffness and equatorial X-ray diffraction, showed that under the conditions studied, crossbridge attachment is stronger since ionic strength has to be increased much more than in relaxed fibres to observe detachment if detachment occurs at all. Furthermore, the attachment and detachment rate constants appear about three orders of magnitude slower as judged from the effect of speed of stretch on apparent fibre stiffness.

Physiological significance of the rate-limiting step of the in vitro *actomyosin ATPase cycle*

The second major question we were interested in is the correlation of the rate-limiting step of the *in vitro* system with a step of the crossbridge cycle *in vivo*.

Judging from the location of the rate-limiting step *in vitro*, this step determines the transition from the weak-binding states to the strong-binding states when the system is cycling. Since it is believed that the strong-binding states represent the main force-generating states and assuming analogy between *in vitro* and *in vivo* system, the step in the crossbridge cycle which is equivalent to the rate-limiting step in solution, might be the step which determines the rate of force generation for cycling crossbridges in active muscle.

For an ideal measurement of the transition from the weak-binding states to the strong-binding states, we have to start with everything being in the weak-binding states and then follow the transition into the main force-generating states by measuring the increase in isometric force. The obvious way of using a relaxed fibre and suddenly unblocking the transition from weak to strong by adding Ca^{2+} cannot be applied since slow processes during activation of the contractile system might dominate the observed rate of force development. The transition from weak to strong itself could be much faster. Therefore, an alternative approach was designed which does not involve activation processes, but allows the piling up of crossbridges in the weak-binding states while the contractile system is fully activated [Brenner, *Biophys. J.* **45**, 155a (1984)].

In earlier experiments designed to study crossbridge attachment during isotonic shortening, it was

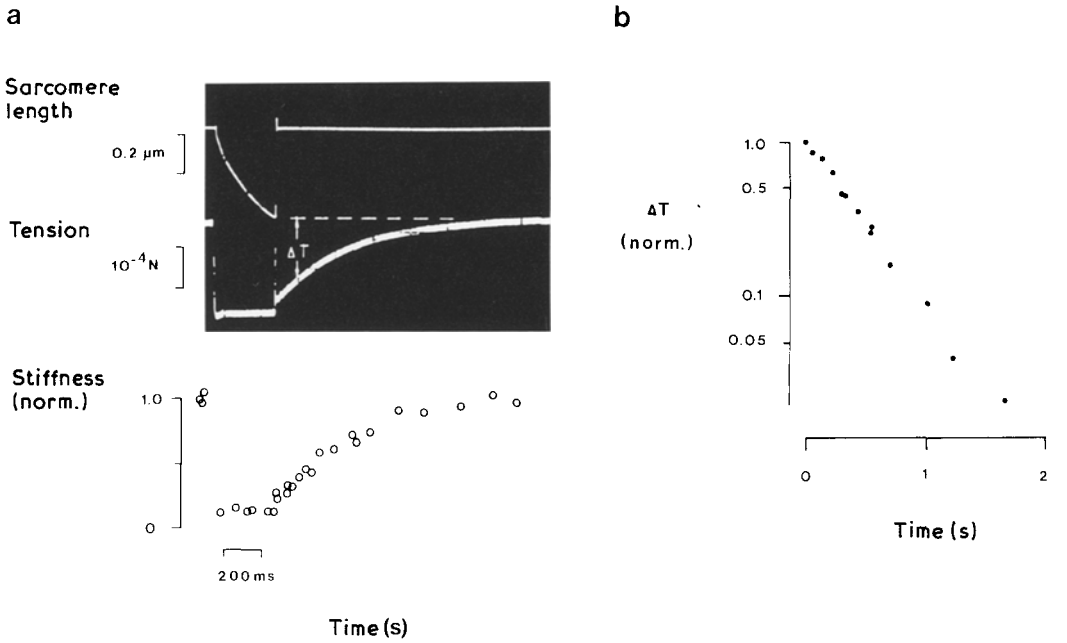

Fig. 2. Force redevelopment after isotonic shortening. (a) Experimental procedure; Top trace, sarcomere length; middle trace, tension; bottom, apparent fibre stiffness. Each open circle represents a single stiffness measurement. Apparent fibre stiffness is measured as the slope when force is plotted against change in sarcomere length during rapid stretches, approximately 10^4 (nm half-sarcomere^{-1}) s^{-1}. The fibre is cycled between isometric contraction and isotonic shortening at low load, approximately 3% of isometric tension (Brenner, 1983). Force redevelopment is recorded after the period of isotonic shortening. To reset the original isometric overlap fibres are restretched to their initial isometric sarcomere length at the end of isotonic shortening. (b) Approximation of force redevelopment by a single exponential function. The difference between steady-state isometric force and instantaneous force during the period of force redevelopment, ΔT as indicated in (a), is plotted against the time after the restretch to reestablish the original overlap. The fairly linear relation when a logarithmic scale is used for ΔT indicates that force redevelopment can satisfactorily be described by a single exponential function.

Single skinned rabbit psoas fibre. l, 7.7 mm; cross-section 80 × 50 μm; temperature, 5° C; ionic strength, 170 mM; pCa about 4.5.

found that in skinned rabbit psoas fibres throughout the period of isotonic shortening, fibre stiffness is always low, only about 10–20% of the isometric stiffness when speed of shortening is at its maximum [Fig. 2a; Brenner, *Biophys. J.* **41**, 99–102 (1983)]. The experiments were done under conditions where no significant fibre stiffness is detectable in relaxed fibres; i.e., under conditions where fibre stiffness cannot be detected arising from attached crossbridges in the weak-binding states. Therefore, the measured fibre stiffness should only reflect crossbridges in the strong-binding states, presumably the main force-generating states. Thus, the decreased fibre stiffness indicates that during isotonic shortening with maximum speed the number of crossbridges in the main force-generating states is reduced to about 10% of the isometric value, i.e. isotonic shortening apparently leads to piling up of crossbridges in the weak-binding states. The recovery of force when isotonic shortening is stopped should therefore reflect the increase in number of crossbridges in the main force-generating states. This is supported by the recovery in fibre stiffness proportional to isometric force during the period of force redevelopment (Fig. 2a). To avoid complications arising from various degrees of filament overlap during the period of force redevelopment, the original overlap was reset by restretching the fibres out of isotonic shortening to their original isometric sarcomere length (Fig. 2a). Since even small amounts of internal shortening significantly slow down the force redevelopment (internal shortening of only 2% results in a delay of about 150 ms at the time of 50% of the full redevelopment under the conditions used in Fig. 2), meaningful experiments have to be done with precise sarcomere length control. This was achieved by monitoring the position of the first order diffraction maximum of laser diffraction patterns and using this parameter as the control signal in a servo system.

To demonstrate that the force redevelopment is closely described by a single exponential function, the difference between instantaneous force and force during isometric steady state (ΔT in Fig. 2a) was plotted against the time after restretch, using a log scale for ΔT. The fairly linear relation (Fig. 2b) indicates that force redevelopment can satisfactorily be described by a single rate constant (Brenner, 1984).

The rate constant of force redevelopment was measured at different temperatures and at ionic strengths of 170 mM and 50 mM. The data are summarized in Table 1. The data at 25° C and 35° C were measured without laser feedback since, at these higher temperatures, the laser-controlled feedback could no longer be used due to deterioration of the laser diffraction pattern. The rate constants measured at these temperatures are therefore too small, being decreased by at least 50%. The rate constants of the actomyosin ATPase, measured with S1 crosslinked to actin [Mornet *et al.*, *Nature, Lond.* **292**, 301–6 (1981); Eisenberg, unpublished data], are the same, within a factor of two, as the rate constants of force redevelopment. These data suggest that the rate-limiting step of the *in vitro* system represents the step which mainly determines the rate of force generation for cycling crossbridges in active muscle (Brenner, 1984).

Table 1. Rate constant of force redevelopment (k) at various conditions.

	Ionic strength	
Temperature	50 mM	170 mM
5° C	2–3	2.5–5
15° C	–	10–15
25° C	–	~30
35° C	–	~60

All rate constants in s^{-1}. Rate constants at 25° C and 35° C measured with overall length control, all others with sarcomere length control during force redevelopment.

In summary, crossbridge attachment in relaxed fibres especially at low ionic strength was demonstrated. Second, these crossbridges appear to be in rapid equilibrium between attachment and detachment, and third, this equilibrium is shifted toward detachment when ionic strength is increased. Together with the experiments in the presence of $MgPP_i$, we therefore conclude that in muscle, as in solution, it is very likely that two groups of crossbridge states exist, weak-binding states and strong-binding states. Furthermore, crossbridge attachment in relaxed fibres suggest that, again as in solution, a major mechanism of regulation is blocking the transition into the main force-generating states while in the absence of Ca^{2+}, i.e., in relaxed fibres, crossbridge attachment is still possible. Finally, the physiological significance of the rate-limiting step in solution is that this step apparently determines the rate of force generation of cycling crossbridges in active muscle. In fact, the magnitude of the measured rate constant agrees well with the rate constant for force generation that is necessary to simulate the experimental force–velocity relation on the basis of a

Huxley-type crossbridge model [Huxley, *Prog. Biophys. biophys. Chem.* **7**, 255–318 (1957)].

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P_i release and the power stroke of the skeletal muscle crossbridge cycle

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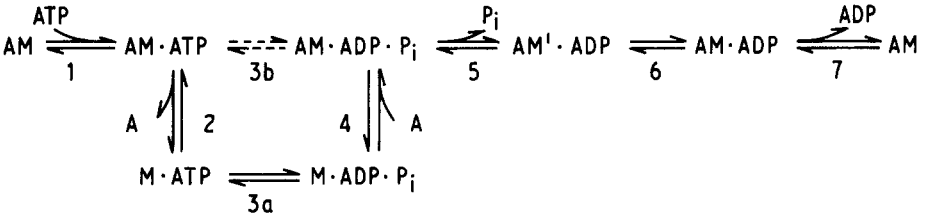
Our working hypothesis for the sequence of chemical reactions which occur during ATP hydrolysis by actomyosin is shown in Scheme 1. This scheme is derived from biochemical studies in many laboratories with the isolated proteins. We expect that in muscle fibres some of the reactions will be controlled by geometrical constraints of the filament lattice and by crossbridge stress and strain.



Scheme 1. A = Actin M = Myosin P_i = Orthophosphate

The experiments described were designed to measure some of these reaction rates in muscle fibres [Goldman *et al.*, *Nature, Lond.* **300**, 701–5 (1982)]. Single glycerol-extracted muscle fibres from rabbit psoas muscle were put into a rigor contraction (AM) by removing ATP. The bathing medium contained 'caged ATP', an inert compound that can be photolysed by a pulse of ultraviolet (347 nm) laser light, to ATP and a by-product. The sudden increase in ATP concentration within the fibre relaxed rigor tension in the absence of Ca^{2+} via reactions 1 and 2. These steps are rapid, independent of the Ca^{2+} concentration, and similar in rate to the corresponding reactions in experiments with the solubilized proteins [Goldman *et al.*, *J. Physiol., Lond.* **354**, 577–604 (1984)]. In photolysis experiments with Ca^{2+} present, reactions 3, 4 and 5 were also found to be rapid compared to the ATP hydrolysis rate of crossbridges during steady active contractions [Goldman *et al.*, *J. Physiol., Lond.* **354**, 605–24 (1984)].

Addition of 10 mM P_i to the bathing medium reduced active tension, but when contraction was initiated by photolysis of caged ATP, the approach to the steady active tension level was more rapid with P_i present than in 0 P_i. In the absence of Ca^{2+}, the final relaxation initiated by caged ATP photolysis was accelerated by addition of P_i to the medium. These results suggest that P_i binds to the crossbridge reversing reaction 5 [Hibberd *et al.*, *Science, N.Y.* **228**, 1317–9 (1985)]. Measurements of exchange between ^{18}O-labelled P_i and solvent H_2O during ATP hydrolysis mediated by activated skinned fibres also showed that P_i binds to the active site on the myosin head and that reactions 4 and 3 are reversible. P_i does not bind effectively to AM·ADP formed by adding ADP to rigor fibres. P_i seems to bind only to a state (AM'·ADP) formed during ATP hydrolysis and crossbridge cycling (unpublished work).

Orthovanadate (V_i), an analogue of P_i, similarly does not bind effectively during rigor with or without ADP or during relaxation. V_i binds to the crossbridges (presumably in the AM'·ADP state) during active cycling and inhibits contraction by forming a stable M·ADP·V_i complex, trapping ADP and V_i within the fibre. Attachment of M·ADP·V_i to actin promotes V_i and ADP release. Binding of V_i or P_i to the AM'·ADP state reverses the power stroke of the crossbridge cycle suggesting that AM'·ADP bears

mechanical force during active contractions [Dantzig & Goldman, *J. gen. Physiol.* (in press; 1985)].

Several of the elementary reactions of the ATPase mechanism, namely ATP binding, crossbridge detachment, ATP hydrolysis, and crossbridge reattachment occur with similar kinetics in a muscle fibre as with the isolated proteins. Other reactions, namely release of P_i and ADP (not discussed) from the actomyosin–products complex, have very different kinetics in fibres. This difference links the product release steps to crossbridge force generation and physiological control of energy transduction in muscle.

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Oxygen-exchange studies on insect fibrillar flight muscle

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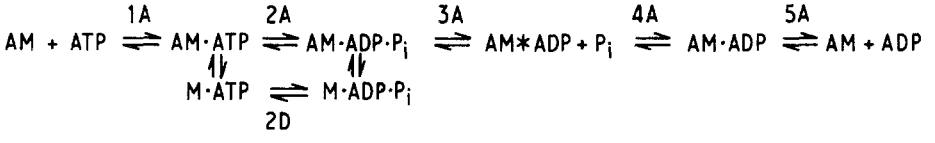
We have used phosphate–water oxygen-exchange to probe the ATPase reactions occurring in glycerol-extracted muscle fibres from the dorsal longitudinal flight muscles of the giant water bug *Lethocerus indicus*. The ATPase activity is much increased by applying small strains and further enhanced by applying small sinusoidal length oscillations [reviewed by Tregear, R. T., In *Handbook of Physiology*, Section 10, *Skeletal Muscle* (edited by PEACHEY, L. D., ADRIAN, R. H. and GEIGER, S. R.), pp. 487–506. Baltimore: Williams and Wilkins (1983)]. Two types of experiment were done to investigate the ATPase reactions under these activated conditions.



Scheme 1.

In the first type of experiments, fibres were incubated with ATP solutions in $D_2{}^{18}O$. When ATP is cleaved (steps 2A or 2D in Scheme 1), one water oxygen (^{18}O) is incorporated into the P_i. If the cleavage step is reversible and P_i can rotate in the catalytic site, oxygen exchange can occur between the phosphate and water, due to multiple reversals of the cleavage [Webb & Trentham, In *Handbook of Physiology*, Section 10, *Skeletal Muscle* (edited by PEACHEY, L. D., ADRIAN, R. H. and GEIGER, S. R.), pp. 237–55. Baltimore: Williams and Wilkins. (1983)]. The distribution of ^{18}O in the P_i was measured with a mass spectrometer and Fig. 1 shows that this intermediate exchange does occur for the conditions studied. To fit the distributions of oxygens for the free fibre to this mechanism of exchange requires two hydrolysis pathways with different kinetics, as is also found with rabbit skeletal fibres [Hibberd *et al.*, *J. biol. Chem.* **260**, 3496–50 (1985)]. However, either stretching or oscillating the fibre results in a single hydrolysis pathway, shown by the distribution of exchanged oxygens. The hatched histograms in Fig. 1 show the best fit theoretical distributions, obtained with the R values indicated. The theoretical derivations are given by Webb & Trentham [*J. biol. Chem.* **256**, 10910–16 (1981)]. It is likely that stretch activation causes some change in the crossbridge cycle, thereby altering internal kinetics of the ATPase to produce these changes in exchange properties. The results cannot be explained *simply* in terms of recruitment of more crossbridges, since the distribution of ^{18}O changes with the different degrees of activation.

We have previously shown that the presence of P_i can have significant effects on the physiological properties of insect flight muscle [White & Thorson, *J. gen. Physiol.* **60**, 307–36. (1972)]. Oxygen exchange offers a direct probe to test P_i binding to AM*ADP in Scheme 1. If such binding occurs, followed by reversal back to the cleavage step, the bound P_i can undergo oxygen exchange by the mechanism outlined above. On release, the P_i would then contain at least one solvent oxygen. To test this, in the second type of experiment, fibres were incubated in Ca^{2+}-activating ATP solutions, also containing 10 mM

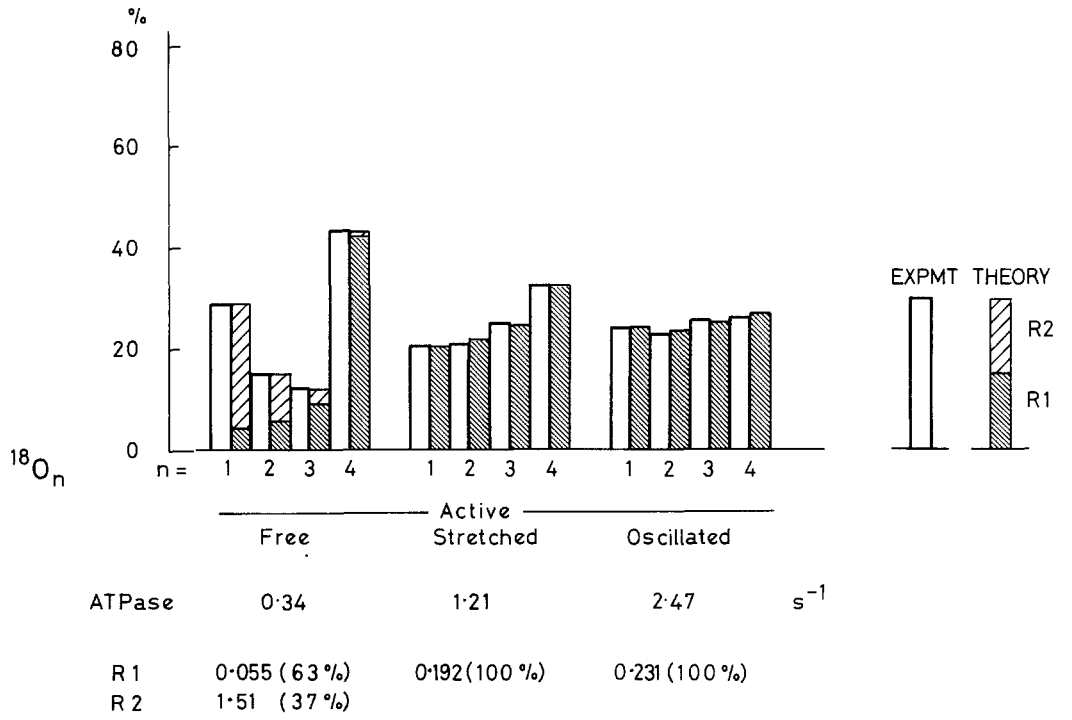


Fig. 1. Analysis of the ^{18}O-content of P_i released from ATP in a series of three experiments in which bundles of five fibres were incubated in an activating solution containing 10 mM $MgCl_2$, 5 mM EGTA, 15 mM KCl, 20 mM histidine, 10 mM Na_2ATP, 1 mM NaN_3, 10 μM diadenosine pentaphosphate, pH 7.0, made up in $D_2^{18}O$, 20° C. The fibres were at zero tension, stretched 3% above rest length or oscillated with an amplitude of 1.5%. The P_i was analysed by mass spectrometry for the proportions of molecules containing 1, 2, 3 or 4 ^{18}O. These proportions are shown in the histograms. The hatched histograms adjacent to the experimental results are described in the text. In these experiments we have used chemically skinned fibres from the giant water bug *Lethocerus indicus*. Details of the skinning procedure and of the mechanical apparatus are given in White [*J. Physiol. Lond.* **343**, 31–57 (1983)]. The fibres were used within four weeks of extraction.

Table 1. Medium exchange.*

Solution	*Condition*	*ATPase* s^{-1}	k_{-3A} $M^{-1}s^{-1}$
Activating	Oscillated	1.77	109
	Strained 3%	2.22	118
	Free	0.21	19
Relaxed	Free	0.032	0.9

*Insect flight muscle fibres incubated in $(^{18}O_4)P_i$

$(^{18}O_4)P_i$. The rate of loss of ^{18}O was determined, to give a measure of the rate of binding. The apparent second order rate constants are shown in Table 1. In all three cases P_i does bind at a significant rate relative to concomitant ATP hydrolysis. The rate constants obtained with strained or oscillated fibres are similar to those obtained with active rabbit fibres [Webb *et al., Biophys. J.* **47**, 60a (1985)], but the free fibres have a much lower rate constant. Three possible explanations are (1) recruitment of more crossbridges in the more active fibres, (2) a change in the rate constant for P_i binding which parallels the change in the ATPase activity and (3) a change in the concentration of AM*ADP which parallels the change in ATPase activity. The last possibility is consistent with the level of AM*ADP, a state implicated in force generation in rabbit fibres [Hibberd *et al., Science* (in press; 1985)], reflecting the level of tension in the fibres.

The molecular structure of troponin C from turkey skeletal muscle at 2.8 Å resolution

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Crystals of turkey skeletal muscle troponin C were grown from $(NH_4)_2SO_4$ solutions at pH 5.0 [Herzberg *et al., J. molec. Biol.* **172**, 345 (1984)]. The crystal structure was solved by the application of the method of multiple isomorphous replacement using 11 heavy atom derivatives. The resulting figure of merit was 0.90 and the electron density map at 2.8 Å resolution was readily interpretable in terms of the molecular structure [Herzberg & James, *Nature, Lond.* **313**, 653–9 (1985)]. Troponin C consists of two domains connected by a nine-turn α-helix. The distance between the centres of mass of the two domains is ≃ 40 Å and there are no direct intramolecular contacts between the domains. In the present crystal structure, two Ca^{2+} ions are bound only in the Ca^{2+} binding loops of the C-terminal domain (referred to as the Ca^{2+}, Mg^{2+} domain). The two Ca^{2+} binding loops in the N-terminal domain have no metal ions present. The Ca^{2+}, Mg^{2+} domain adopts the molecular conformation of the CD, EF domain in parvalbumin with interhelix angles of 107° (the angle between the axes of helices E, F and G, H). The N-terminal domain is specific for binding Ca^{2+}. The molecular conformation of this domain is distinctly different from that of the Ca^{2+}, Mg^{2+} domain. The interhelix angles (between helices A and B and between C and D) are much larger, averaging 147°. In addition, there is a fifth helix in the Ca^{2+} specific domain that is formed by the first 12 residues of the molecule. The packing of this helix and helices A, B and C of the N-domain is such that the residues of helix D are predominantly hydrophobic and two helical turns are completely buried in the hydrophobic core of this domain. Analysis of this molecular structure led us to propose that it is similar to the structure of TnC in the relaxed state of muscle. One of the possible conformational changes occurring when Ca^{2+} binds to the regulatory N-domain is a relative shift of the helices A, B and C so that the conformation of this domain resembles more the conformation of the C-domain. Such changes will also involve those residues that form the loop regions of the two potential Ca^{2+} binding sites in the N-domain.

The long nine-turn α-helix that runs between the N-terminal domain and the C-terminal domain has a central region of 11 residues of which eight are charged (Lys, Glu, and Asp). The central residue is Gly92 a residue not normally found in α-helices. This suggests conformational flexibility of the long helical linker that may be associated with Ca^{2+} binding in the regulatory domain. The structure of TnC in relation to regulation of muscle contraction will be best appreciated from the structure of complexes with other components of troponin and the members of the thin filament.

Packing analysis of crystalline myosin subfragment-1: implications for the structure of the head

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Crystals of myosin subfragment-1 (S1) from avian skeletal muscle have been grown reproducibly [Rayment & Winkelmann, *Proc. natn. Acad. Sci. U.S.A.* **81**, 4378–80 (1984)]. They have been examined by X-ray diffraction and electron microscopy to determine how the molecules pack in the unit cell and to gain preliminary information on the size and shape of the myosin head [Winkelmann *et al., J. molec. Biol.* (in press; 1985)].

S1 crystallizes in space group $P2_12_12_1$ where a = 107 Å; b = 117 Å and c = 278 Å. The crystals diffract to at least 4.5 Å resolution. Analysis of the X-ray diffraction photographs shows that there are eight molecules in the unit cell with two in the asymmetric unit related by a non-crystallographic or local two-fold axis. These axes are oriented almost parallel to the a axis and located approximately 4.3 Å in the c direction away from the crystallographic two-fold screw axis that run parallel to a. As a consequence of this organization, two molecules of myosin S1 lie almost directly on top of one another, in projection down the a axis, except for a translation of about 9 Å relative to each other along c. This packing arrangement restricts the thickness of the myosin head in the a direction in the crystal to be no more than half the length of a axis i.e. < 54 Å.

Small crystals were fixed and embedded in the presence of tannic acid [Akey & Edelstein, *J. molec. Biol.* **163**, 575–612 (1983)] and thin sections cut perpendicular to each of the three major crystallographic axes. In this method of fixation, the solvent regions of the crystals take up the stain giving rise to a negatively stained thin section in contrast to other methods of fixation. The computed transforms from these thin sections show a remarkable similarity to the low resolution X-ray diffraction patterns. Consequently, the stain distribution observed in the electron micrographs reflects the solvent distribution in the native hydrated cyrstals. Image analysis of these micrographs confirms the packing arrangement deduced from X-ray diffraction and gives the approximate size, shape and orientation of the molecule in the crystal lattice. They show that the

molecule, which has a tadpole-like shape, is at least 160 Å long with a maximum thickness of about 60 Å, and that it has a marked curvature in the unit cell.

These preliminary studies, apart from establishing a definite minimum length for the head, will be of great value in interpreting the mass distribution in decorated F-actin and native thin filaments. A three-dimensional reconstruction of the negatively stained thin sections is in progress. The orientation and location of the molecules in the unit cell derived from these studies will also be an enormous help in locating the positions of heavy atom substitutions in isomorphous derivatives. At this time, two potential heavy atom derivatives have been prepared which will be analysed in further detail once larger crystals have been obtained.

Crystallographic studies on the profilin–actin complex

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Actin is found in nearly all eukaryotic cells where it participates in a wide variety of functions where movement or force is required [dos Remedios & Barden, In *Actin: Its Structure and Function in Muscle and Non-muscle Cells*. Sydney: Academic Press. 1983)]. As a major component of most cytoskeletons it forms part of the dynamic, rapidly changing, leading edges displayed by motile cells. In its filamentous form it can be gathered into ordered bundles as in the microvilli of the inner intestinal lumen, cell surface microspikes, in the acrosomal secretion of sea urchin sperm, or the stereocilium of the inner ear. Of course, in skeletal muscle it is the principal structural protein in the thin filament which forms part of the interdigitating filament system responsible for force generation. In many nonmuscle cells actin is found in a 1:1 complex with a small (15 kDa) basic protein named profilin whose role seems to be to maintain actin in the monomeric state until some signal arrives to trip the equilibrium towards the filamentous state. This process has been directly demonstrated for platelets where stimulation by platelet derived growth factor (PDGF) leads to the separation of profilin from actin and rapid filament formation. The precise details of this transmembrane signalling system are unknown. Lindberg and co-workers [Lassing & Lindberg, *Nature, Lond.* (in press; 1985)] have, however recently demonstrated that the profilin–actin complex interacts directly with phosphatidyl inositol bisphosphate, a phospholipid present at about the 1% level in the inner leaflet of most plasma membranes. This interaction leads to localization of profilin in phospholipid vesicles with a concomitant release of polymerizing actin. This suggests that binding of growth factors to their cognate receptors may initiate cell movements through specific modifications of phospholipid molecules which are then recognized by profilin–actin.

We have been studying the crystal structure of the profilin–actin complex [Carlsson *et al., J. molec. Biol.* **105**, 353–66 (1976)]. It crystallizes in space group $P2_12_12_1$ with a = 38.6 Å, b = 71.6 Å, c = 187 Å. A significant obstacle in solving this structure has been the extreme variability in the dimension of the c-axis depending on the composition of the crystal soaking medium. For example, an increase in ionic strength or a drop in the pH can lead to a 10% contraction of the unit cell (to c = 170 Å).

Surprisingly, the diffraction limit of the crystal increases from about 2.6 Å to better than 1.9 Å as a result of this 'tightening up' process. More worrying than this polymorphism, however, which in principle can be controlled, is the fact that heavy metal reagents such as mercurials, so important to protein structure determination, lead to large changes in the unit cell. However, conditions were found for preparing stable and useful heavy atom derivatives.

The electron density at 6 Å revealed actin to be an elongated molecule (75 Å × 38 Å × 35 Å) and profilin to be a compact structure (38 Å × 35 Å × 35 Å). These exhibit rather extensive contacts between a single profilin molecule and two actin molecules in the unit cell and, also, significant contacts between actin molecules along the 72 Å two-fold screw axis. It is possible that contacts along this axis are very similar to those in the native F-actin filament. In this case, the two-fold 'ribbon' would need to be twisted by approximately 13° per subunit, accompanied by a change in pitch from 72 Å to 55 Å. Although this may seem large it can be accomplished by relatively small conformational changes in the actin subunit, comparable in fact to those observed in other macromolecular assemblies such as tomato bushy stunt virus [Harrison *et al., Nature, Lond.* **276**, 368 (1978)]. The 'ribbon' structure for actin has not been observed before and would appear to be a consequence of the particular forces present in the crystal when profilin is complexed to actin.

We have also located the position of an ATP molecule in the crystal. It is situated near a sulphydryl group, which we believe to be Cys 374 on a neighbouring actin molecule in the crystal. This may not be the native ATP binding site, however, because (1) profilin is known to weaken the binding by actin of ATP, and (2) ATP is thought to be in an internal cleft formed by two domains in the actin structure deduced at 6 Å from the crystalline DNase–actin structure [Suck *et al., Proc. natn. Acad. Sci. U.S.A.* **78**, 4319–23 (1981)].