THE ROYAL SOCIETY

Past, present and future experiments on muscle

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Since the basic outline of the sliding filament mechanism became apparent some 45 years ago, the principal challenge, an experimental one, has been to produce definitive evidence about the detailed molecular mechanisms by which myosin cross-bridges produce force and movement in a muscle. More recently, similar questions could be posed about other molecular motors, in non-muscle cells. This problem proved unexpectedly difficult to solve, in part because of the technical difficulty of obtaining the structural and mechanical information required about rapid events within macromolecules, especially in a working system, and this triggered many remarkable technical developments. There is now very strong evidence for a large change in shape of the myosin heads during ATP hydrolysis, consistent with a lever-arm mechanism. Whether this does indeed provide the driving force for contraction and movement—and, if so, exactly how—and whether some other processes could also play a significant role, is discussed in the light of the experimental and theoretical findings presented at this meeting, and other recent and long-term evidence.

Keywords: contraction; molecular mechanism; structure; X-ray

1. INTRODUCTION

The problem of how muscles contract has been a surprisingly long-lived one. It is a remarkable fact that, continuously over the past 50 years, since The Royal Society Discussion Meeting organized by A. V. Hill in 1949 (Hill 1950), the field has been one of considerable intellectual excitement, with important and rewarding progress being made all the time in experiments and understanding. Yet the ultimate objective of our work, though its nature was clearly identified 45 years ago, turned out to be much further away than was originally realized. There was much more that needed to be discovered than first met the eye. However, the intervening pathways have been full of interesting challenges, and have led to insights into a much broader range of motile phenomena than one had originally dared to hope. So the momentum and motivation have been maintained.

This present meeting, and the situation in the field that it reflects, has some of the same characteristics. We have all this marvellous new crystallographic evidence, at high resolution, about the structural changes that can take place in the head region of the myosin molecule during ATP hydrolysis, yet the task of proving that these occur in the actomyosin complex in muscle—and must develop force—is still not completed, despite a wealth of circumstantial evidence. Indeed, even though the tilting lever-arm mechanism is now remarkably well supported, there still seems to me to be room for some additional process to be involved as well. And reducing all this to basic physics and an even approximate set of equations is clearly some distance away.

As to the relationship between the linear motor mechanisms in myosin and kinesin, and the remarkable rotary motors now being characterized, there is little I can add to what has been discussed at this meeting, and the detailed mechanisms do not seem to be closely related. However, I would like to comment on the lengthy chain of experimental evidence on the muscle mechanism and the reasoning that has led us to the present position, and the further evidence that we need to obtain.

2. THE EARLY YEARS

At the time of the 1949 discussion meeting on muscle, it was recognized in a general way that contraction involved the interaction of actin and myosin, two proteins that were still poorly characterized, and that this interaction was expressed through the shortening of a longitudinally orientated filament structure in muscle. It was suspected that ATP hydrolysis was involved, but exactly how was still uncertain. There was no concept that individual myosin molecules might function as individual molecular motors. Indeed, as an outsider coming into the field from physics, I was amazed at how little was known—or even thought—about the underlying molecular events.

The overlapping filament model, with actin and myosin each in their own filaments, in separate but interacting arrays, provided the first clear picture of the structure, from a combination of X-ray diffraction, phase-contrast light microscopy and electron microscopy (Hanson & Huxley 1953; Huxley 1951, 1952, 1953*a*,*b*). (Electron microscopy was then just beginning to reveal the remarkable structures of the submicroscopic world, but evidence from such observations was sometimes regarded rather sceptically!) The overlapping filament

array model soon developed into the sliding filament model, based on changes in the visible band pattern during contraction and stretch (Huxley & Niedergerke 1954; Huxley & Hanson 1954) and on other evidence that individual filaments were approximately constant in length. It was suggested that sliding was produced by the action of cross-bridges between actin and myosin filaments, structural elements whose existence had been postulated a couple of years earlier on the basis of the X-ray results and the inextensibility of muscle *in rigor* (Huxley 1952), and which were detected in muscle cross-sections by electron microscopy the next year (Huxley 1953*b*). They were clearly demonstrated using the electron microscope during 1955–1957 (Huxley 1957; Huxley & Hanson 1956) and were identified as projections from the myosin filaments; in one model they were thought to move backwards and forwards in some cyclical way (Hanson & Huxley 1955).

It took a long time (about 15 years, until around 1970) and a lot of further work for this general model to become moderately well accepted (though not universally so). The crucial factors were the elucidation of a great deal of detail about the structure of the actin and myosin filaments, which all fitted in with the requirements of the model (Worthington 1959; Hanson & Lowy 1963; Huxley 1963; Elliott 1964; Elliott *et al*. 1967; Huxley & Brown 1967), the recognition that many general properties of muscles could be explained by the model (Huxley 1960; Huxley & Hanson 1960) and particularly the theoretical demonstration (A. F. Huxley 1957), that a particular version of the model—essentially a thermal ratchet—could account in a very convincing way for a good deal of the detailed behaviour of vertebrate striated muscle.

In the early days, we had thought in terms of cross-bridges tilting back and forth on the myosin filaments, i.e. being firmly attached laterally to the myosin filament backbone. However, the X-ray finding that filament separation could vary without apparently interfering with the actin–myosin interaction led us to think of a rather different model (Huxley 1969). In this, the force-generating part of the structure is located in the attachment of the myosin head to the actin filament, and longitudinal force generated by tilting of the head on actin is transmitted to the myosin filaments via the S2 portion of myosin. The S2, while longitudinally rigid under stretch, was thought to provide the required radial and azimuthal flexibility.

This model was taken up, and developed further in the A. F. Huxley & Simmons (1971) mechanism, with several stepwise changes in the angle of attachment of the myosin head to actin (for example), and an elastic component in each cross-bridge, possibly in the S2 element. This model could account for the remarkable rapid mechanical transients, which they had characterized in single muscle fibres, and which were not easy to explain satisfactorily on the original thermal ratchet mechanism.

Around the same time, Lymn & Taylor (1971) showed, by ingenious enzyme kinetic and stopped flow experiments, that the hydrolysis of ATP by actomyosin followed a very unexpected pathway, but one in which dissociation and reassociation of myosin and actin took place during each ATPase cycle, exactly as was required by the sliding filament model.

3. THE MATURE MODEL

Thus the status of the problem then (1969–1971) was that we had, as a result of the previous 20 years' work, a very good idea of the overall structure of a muscle, which proteins were involved and approximately how they were arranged; and we knew how the overall structure behaved in contraction—basically, sliding at constant filament length. We had plausible models involving cyclically operating myosin cross-bridges tilting backwards and forwards and attaching to and detaching from actin at appropriate points in the ATPase cycle. But we had absolutely zero experimental evidence that such cross-bridge movement actually did take place. Clearly, such evidence was essential.

The obstacles to obtaining this kind of information have taken a long time to overcome—about 30 years so far, and we have still a little way further to go—in large part because the necessary technology, both physical and biological, has taken many years to reach the required level.

The first step was to show that the cross-bridges moved at the onset of activity. This was accomplished by X-ray diffraction using laboratory X-ray sources (rotating anode tubes) in the late 1960s and early 1970s (Haselgrove & Huxley 1973; Huxley & Brown 1967; Huxley & Haselgrove 1976), when it was shown that the myosin heads moved towards actin as soon as a muscle was activated, and that they also lost the regular helical arrangement present in resting muscle during contraction and became much more disordered. However, it was surprising and disappointing to find that no striking 'labelling' of the actin pattern developed as the myosin layer-line pattern disappeared during contraction. The relatively small changes that were seen in the visible actin reflections (at 59 Å and 51 Å) could not be interpreted in the absence of detailed knowledge of the structures of the actin and myosin molecules.

But the real problem was to see what changes (if any) there were during the postulated working strokes of the cross-bridges. These are normally completely asynchronous during contraction, and this makes informative measurements much more difficult to perform. The Huxley & Simmons (1971) quick-release manoeuvre showed us how to partially synchronize the movements (if indeed they took place), but the difficulty was how to detect them externally in a working muscle and how to do so during the millisecond or two during which some synchrony can be maintained.

Simmons and I were able to see changes in the intensity of the 143 Å meridional X-ray reflection (which comes from the cross-bridge repeat) using synchrotron radiation as an intense X-ray source (Huxley *et al*. 1981, 1983), and these changes were found to be closely synchronized with 1 ms releases or stretches of an intact contracting muscle. This experiment showed that some type of major structural change, in an axial direction, was happening in the cross-bridges during their working strokes; this could be the postulated tilting, but that was not the only possible explanation. The change in intensity was a large one, despite the relatively small proportion (20% or less) of myosin heads that were believed (from X-ray and other evidence) to be generating force at any

moment. Presumably, the other heads were too disordered to contribute.

At that time, this was the only useful reflection intense enough to study adequately with the necessary high time resolution, and the equatorial reflections showed virtually no change, during a quick release.

The other general approach was to attach spectroscopic labels of various kinds to the myosin and look for preferred orientations and changes between different states and in muscle transients (Thomas & Cooke 1980; Yanagida 1981). Unfortunately, for many years the results from these techniques were somewhat misleading, because whatever label was used, they seemed to show only a single orientation in the attached myosin heads, and no change of orientation in a quick release. This was bad news for the tilting cross-bridge model and sowed a certain amount of doubt about the whole sliding filament theory, despite all the earlier evidence. There was even a scurrilous article in *Nature*, jeering at the supposed imminent demise of yet another outdated theory!

4. THE MODERN ERA

The situation was partially rescued by the arrival of the *in vitro* motility systems, in which actin filaments could be clearly seen, in the light microscope, sliding over isolated myosin heads on a surface (Kron & Spudich 1986; Toyashima *et al*. 1987). The problem was further clarified when the high-resolution myosin head structure was published in 1993 (Rayment *et al*. 1993*a*), and it was apparent that all of the previous spectroscopic labels had been placed on the so-called 'catalytic domain', which could remain bound to actin in a fixed orientation, while the long protruding 'regulatory domain', which was clearly likely to act in some way as a tilting lever arm, had not been labelled. The atomic structure of the actin filament had now also been solved (Holmes *et al*. 1990), and so it was possible to have a very good idea of how the myosin head would bind to it (Rayment *et al*. 1993*b*).

Since then, very elegant experiments by Irving, Goldman and Trentham, and their collaborators (Hopkins *et al*. 1998; Irving *et al*. 1995), have attached fluorescent labels to specific sites on the lever arms by very specific techniques, and have measured changes in their orientation, which vary qualitatively in exactly the expected way in the course of rapid releases and stretches of the labelled, single muscle fibres during ATP-induced contraction. However, the average change in angle is quite small, just 3–5°. The problem is that the signals from all the labelled cross-bridges in a muscle are being averaged together, and if, as seems likely from internal evidence in these experiments and from other work, only a small proportion of them are actively developing tension at any given moment, then one is having to look at a small signal on a high background and the actual change in angle could be much greater.

Also in recent years, Irving and Lombardi, and their colleagues (Dobbie *et al*. 1998; Irving *et al*. 1992; Lombardi *et al*. 1995), have been able to make superb high time-resolution measurements on the 143 Å meridional X-ray reflection from single muscle fibres during mechanical transients, and have shown in great detail that a tilting lever-arm model with a change in angle of 30° or more can account for all the effects seen in this reflection in a variety of mechanical transient manoeuvres. The advantage of this X-ray method, as mentioned above, is that it appears (and this is supported by other recent evidence) that in a contracting muscle, most of the 143 Å signal comes from the tension-generating cross-bridges, and that the ones that are 'between engagements' are axially disordered and contribute very little to the measured signal.

However, the corresponding disadvantage is that this is a myosin periodicity, presumably visible because myosin heads need only move a relatively small distance axially (probably $< \pm 27$ Å) from their average 143 Å repeating position to find an actin monomer to which to attach. In reality, however, it is the myosin heads that are specifically attached to the actin periodicity that we are interested in, so it is the actin reflections, especially the off-meridional layer-lines, which should in principle be the more direct source of information, and which should show which part of the myosin head is moving. However, the corresponding disadvantage is that the actin signal will be diluted by the unoccupied actin sites.

Until recently, these actin reflections were too weak to measure at millisecond time resolution. However, due to continued improvements in technology, and the use of an undulator beam line on the APS Storage Ring at Argonne, we have now been able to get good two-dimensional data on these reflection with millisecond time resolution (Huxley *et al*. 1999). Use of charged-coupled device cameras, and also imaging plates, makes it possible to employ the full available flux, and particularly strong recordings can be made using a synchronized succession of 2 ms time windows and quick release and restretch cycles during each tetanic contraction. The patterns do indeed show characteristic changes in several of the actin reflections during a quick release, which is a significant advance. However, getting a good match between the observed layer-line profiles, and those calculated from the full atomic structures of actin and myosin inserted into the 'decorated actin filament' structures believed to be present in contraction, is not straightforward, and will keep us occupied for some time.

5. THE CURRENT SITUATION

I think it is fair to say that there is now strong evidence, from the X-ray diffraction and polarized fluorescence measurements on functioning muscles, that a change in tilt of the so-called 'lever arm' does take place during the working stroke of the myosin cross-bridges. At the same time, there is definitive evidence from X-ray crystallography for two very different orientations of the crystallography of the lever arm relative to the catalytic subunit depending on which nucleotide or analogue is occupying the binding site (see review by Holmes 1998). When these orientations are assigned to their expected position in the ATPase cycle, and are incorporated into the 'decorated' actin structure, then they predict that the actin-attached myosin heads go through the appropriately directed lever-arm tilting movement that would be required between the successive steps of the tilting cross-bridge model. Thus either that model is correct, or it is

being mimicked by some other mechanism in a rather remarkable fashion.

Nevertheless, there is one further point that one should consider. One needs to ask—is this the whole story, or is there perhaps some other process going on as well? Could there be an element of a thermal ratchet mechanism, in addition to the lever-arm system, to take direct advantage of the initial binding energy of myosin to actin? As I, and others, have pointed out elsewhere (Huxley 1998), the measurements from many (not all) of the *in vitro* motile systems seem to be converging on a figure of about 5 nm for the working stroke, and about 5 pN (or less) for the average force. This would give 25 pN nm for the available mechanical energy, or slightly over 6 kT. However, the amount of mechanical energy released in a contracting muscle by one molecule of ATP is much greater than this. Even using a conservative figure of 48 kJ mol^{-1} for the free energy of ATP hydrolysis (Woledge *et al.* 1985)—equivalent to about 19 kT per molecule—the observed value of 60% or more of the energy released in contraction that can appear as mechanical work leads to a figure of about 11.5 kT of work per myosin head per ATP, or almost twice the amount observed in the *in vitro* experiments. Thus, either the force of the distance or both are being seriously underestimated in these experiments, or else a cross-bridge in a working muscle somehow manages to convert another 5 or 6 kT of free energy into mechanical work for each molecule of ATP hydrolysed.

This is an amount of energy which could feasibly be supplied within the short time required for an additional 'stroke' of 4–5 nm by Brownian motion of the myosin heads against an elastic restoring force, as in an A. F. Huxley (1957)-type mechanism. This energy would be balanced by some of the binding energy of myosin to actin. This additional displacement might not show up in many of the *in vitro* experiments, since the myosin filament structure may be required for the head to have the appropriate position and mobility. This may be why Dr Yanagida observes larger steps than many other '*in vitro*' experimenters when he uses synthetic myosin filament backbones to support individual myosin molecules, rather than having them deposited on a surface. And perhaps this type of behaviour is occurring in his recent experiments (Kitamura *et al.* 1999) in which a single myosin head is held in contact with an actin filament bundle, in a mechanical system which has high rigidity in a direction perpendicular to the actin filament and very low rigidity along the filament axis, so that the head can make particularly long Brownian excursions in searching for a preferred actin site, while being unable to diffuse away laterally immediately on each dissociation.

6. THE FUTURE

As far as future experiments are concerned, I think the next urgent needs are (i) to find a way of crystallizing myosin heads in combination with actin, for crystallographic analysis; (ii) to develop the technology and analysis of *in vitro* motile systems even further, so that definitive values of force and step-length can be obtained, at high time resolution and in a situation closely approximating that in muscle; and (iii) to obtain as detailed as possible three-dimensional solutions, at high time resolution, of X-ray diffraction data that can now be obtained from intact muscle during mechanical transients.

A request to the theorists would be to look very closely at the kinds of experimental data that are now available, or could be obtained, and give advice on sophisticated ways of analysing them to distinguish between the different possible types of models that might represent the underlying mechanisms.

Finally, even when all the changes in the atomic structure of the myosin head have been characterized (and in actin too if necessary), and we can see how they are linked structurally to the cross-bridge 'stroke', that is not the end of the problem. One still has to understand quantitatively the detailed internal structural mechanics and energetics of the myosin head and actin interacting with ATP, and I think we are some distance from being able to make those kinds of calculation at the moment—and to check them by genetic engineering. So there is still much to do.

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