SLIDING FILAMENT MECHANISM IN MUSCLE CONTRACTION

Fifty Years of Research
ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

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SLIDING FILAMENT MECHANISM IN MUSCLE CONTRACTION
Fifty Years of Research

Edited by

Haruo Sugi
Teiko University
Tokyo, Japan

Springer
Fifty years have passed since the monumental discovery that muscle contraction results from relative sliding between the thick filaments, consisting mainly of myosin, and the thin filaments, consisting mainly of actin (A.F. Huxley and Niedergerke, Nature 173: 971-973, 1954; H.E. Huxley and Hanson, Nature 173: 973-976, 1954). Until the early 1970’s, considerable progress have been achieved in the research field of muscle contraction. For example, A.F. Huxley and his coworkers put forward a contraction model, in which the myofilament sliding is caused by alternate formation and breaking of cross-links between the cross-bridges on the thick filament and the sites on the thin filament, while biochemical studies on actomyosin ATPase reactions indicated that, in solution, actin and myosin also repeat attachment-detachment cycles. Thus, when a Cold Spring Harbor Symposium on the Mechanism of Muscle Contraction was held in 1972, most participants felt that the molecular mechanism of muscle contraction would soon be clarified, at least in principle.

Contrary to the above “optimistic” expectation, however, we can not yet give a clear answer to the question, “what makes the filaments slide?” Of course, muscle investigators have continuously made every effort to progress their respective research work towards the understanding of muscle contraction mechanisms. Based on the idea that muscle contraction is best studies by using specimens in which the three-dimensional myofilament-lattice structure is preserved, the author has organized six symposia on muscle contraction, each followed by publication of the Proceedings:

*Symposium held as US-Japan Cooperative Seminar organized by H. Sugi and G.H. Pollack.

Except for the first proceedings, the second to the sixth proceedings are published as series of Advances in Experimental Medicine and Biology (No. 170, 226, 332, 453 and 538).

In each publication, I always intended to include records of burning discussions among participants during the meeting, in order that the proceedings could be made as informative and attractive as possible. Fortunately, these proceedings have been widely regarded as milestones showing the progress of research work achieved at the time of each meeting.

This volume presents the proceedings of the symposium, which the author organized in celebration of the fiftieth anniversary of the discovery of the myofilament sliding in muscle contraction. In this connection, it was my great pleasure that I could have Professor Hugh E. Huxley, who was mainly responsible for the monumental discovery, in this meeting. At the beginning of this volume, he describes his exciting experiences during the course of his monumental discovery. The readers of this volume would realize a number of mysteries in this research field still remain despite the extensive studies accumulated in recent fifty years. Indeed, "Nature is much wiser than human being." I heartily hope that this volume would stimulate young investigators to start challenging the molecular mechanism of muscle contraction.

Finally, the author would like to express his thanks to Drs. J.A. Rall, J.W. Squire and S. Winegrad for their generous help in editing the discussion records, and to Mrs. Y. Suzuki for typing manuscripts.

Haruo Sugi
ACKNOWLEDGEMENT

The editor would like to express sincere thanks to the Uehara Memorial Foundation for Life Science and the Inoue Foundation for Science for generous financial support, which made this meeting possible.

The editor also owes a debt of gratitude to Dr. Kazutaka Kobayashi, Ms. Yuka Suzuki and Ms. Ibuki Shirakawa at Teikyo University for their enormous efforts in preparing the discussion records in this volume, and Drs. Teizo Tsuchiya, Takenori Yamada, Shigeru Chaen, Seiryo Sugiura and Kaoru Katoh for their help in carrying out this meeting.
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I. SLIDING FILAMENT MECHANISM AT THE MOLECULAR LEVEL
EARLY DEVELOPMENTS IN MUSCLE RESEARCH
AND THE ROLE OF NEW STRUCTURAL
TECHNOLOGIES

H.E. Huxley

1. INTRODUCTION

A few years after the end of World War II, in a small Medical Research Unit located, somewhat anomalously, in the Cavendish Laboratory (a physics laboratory) in Cambridge, England, a number of scientific discoveries were made which had far-reaching effects on the subsequent course of biological research. The best known of these was of course the proposal of the double helical base-paired structure of DNA, by Watson and Crick, in 1953. But in that same year Max Perutz, the head of the Unit, discovered how to determine the phases of X-ray reflections from protein crystals and thereby how to solve the atomic structure of protein molecules, which he and John Kendrew proceeded to do for haemoglobin and myoglobin during the next few years. This paved the way for the tens of thousands of different detailed protein structures which have now been determined, and, together with the basic knowledge of life processes that flowed from understanding of how DNA functions, has revolutionized biology and medicine in the last half century.

A little earlier than these momentous discoveries, during the years from 1949 through 1952, some other new discoveries were made in the same small unit, ones of a more specialized nature, but ones which did begin to set a new direction for work on the nature of muscle contraction. These discoveries flowed from the application of the same concepts as the two very dramatic ones mentioned above.

These concepts were extremely simple, almost simple-minded ones. The first was that it was very important to know what was the detailed structure of biological molecules and tissues, in the belief that information at the molecular and atomic level would be the essential key to understanding how all these biological processes worked, and that anything else was mere speculation. The second followed directly from this, and it was that methods had to be developed and applied in biology to carry out such structural work, and that a laboratory where this was done had to equip itself to carry out that development on the premises.

The professor of physics who headed the Cavendish Laboratory at that time was Sir Lawrence Bragg, who had invented X-ray diffraction analysis (and received the Nobel Prize for it at the age of 24), and it was he who had supported Perutz for many

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1 H.E. Huxley, Rosenstiel Center (MS-029), Brandeis University, 415 South St., Waltham, MA 02454-9110 USA
years, and who was instrumental in setting up the MRC Unit, run by Perutz and Kendrew, where I was a research student, and where Crick and Watson solved the structure of DNA. The Cavendish Laboratory had a very strong experimental tradition, exemplified in the Pt II Physics Practical Glass, where we used gold-leaf electrosopes to measure radioactive decay and α-particle ranges, learned to blow our own glassware and make cathode ray tubes, and repeat Millikan’s oil drop determination of electronic charge. There was a large well-equipped mechanical workshop in the Cavendish, and a small student’s workshop which our group used, with plenty of odd pieces of metal sheet and tubing, and a mechanic to help us use drills and lathes. And Max always stayed close to the bench!

So when the MRC Laboratory of Molecular Biology was set upon its own a few years later, an essential feature was the provision made for technological development, with a magnificently equipped and staffed mechanical workshop, a large electronic workshop, and smaller workshops elsewhere in the lab. This recognized that the structural work would require the highest level of technical support. The success of the laboratory testified to the strength of that approach.

2. EARLY MUSCLE WORK

For my own part, fascinated by experimental atomic physics, very conversant with the importance of knowledge of atomic structure in understanding so many of the properties of matter, it was a natural and easy transition to accept that detailed structural information was essential to even begin to understand biological processes. Vague theories were no good. And it was clear that new techniques were now waiting to be exploited.

X-ray diffraction was hardly new, but its use in biology was at that time quite limited. However, some years earlier Bernal had discovered that detailed diffraction patterns could be obtained from protein crystals if -- and only if -- they were kept in a fully 'native' environment, hydrated, and in their mother-liquor. This was the foundation of protein crystallography. Another pioneer, Astbury, had looked at the wide-angle X-ray diagram of muscle, and found that there was no discernible change in it during contraction, indicating that the basic polypeptide chain configurations remained the same. So when I first started thinking about how muscles might contract, I got the idea that there must be larger structural units, protein molecules or assemblies of protein molecules, still way beyond the resolution of the light microscope, which interacted with each other and re-arranged themselves in some way so as to cause the muscle to shorten. To see this type of structure, which I thought might show structural repeats upward from fifty to a few hundred angstroms in size, I would need a low-angle X-ray camera, since the reflections would lie within 1° or less of the direct beam, and that might be why they had not been noticed previously.

Also, such cameras had to employ very narrow slits to collimate the X-ray beam, and usually needed to be quite long, to allow the pattern to spread out sufficiently. This meant that the total X-ray flux tended to be small, and the flux per unit area at the detector – film – was even smaller. Furthermore, I thought it was imperative to examine muscle in its native hydrated state, rather than dried down into a more concentrated state, as had been done in some previous work. So all this meant that I would only get extremely weak patterns and have impossibly long exposure times – even some protein crystals were then needing up to a month -- unless I could drastically increase the camera speed.

At that time, in the year 1949, there was a lot of interest at the laboratory in increasing the intensity available from X-ray tubes. One way to do this was to use a rapidly rotating anode, since the basic limitation was the rate at which heat could be dissipated from the incident electron beam, to avoid melting the copper target. This
could be partly overcome by continuously presenting to the beam a fresh copper surface which had had time to cool during the cycle of rotation since its last exposure. Such machines were not easy to construct, since they operated in vacuums, with rotating seals, usually at 40,000 volts potential difference between anode and cathode, needed relatively large currents, and had to have water cooling. The ones which had been built previously were too unreliable for routine use. So a good electronic engineer was hired to construct a usable device, but it became clear that it would not be ready soon enough to help my thesis work.

Another way of increasing the available x-ray flux per unit area emitted by the target was to use a much smaller focal spot for the electron beam. Cooling of the irradiated area takes place laterally around the periphery of the spot, as well as vertically, into the depth of the target. So cooling is much more efficient for smaller or narrower spots, and the permissible flux per unit area in fact increases approximately linearly as the inverse of the diameter of the focus. Ehrenberg and Spear, in Bernal's lab, in London, had built such a microfocus tube, in part for other reasons, and I was fortunate to obtain a prototype through my supervisor, John Kendrew, who had been a wartime colleague of Bernal (and who had been drawn into protein crystallography by him). This tube operated with a 50 μm spot, which would be effectively forshortened to 5μ using a shallow viewing angle (5°). So I had an extremely bright, very narrow source, ideal for low angle diffraction, and was able to construct a camera with correspondingly narrow slits and only a few centimeters specimen to film distance which still gave me order-to-order resolution of several thousand angstroms, and a first order resolution well over 500 Å with relatively high (then!) recording speed. The patterns, recorded on film, needed to be viewed through a low-power microscope.

Looking for equatorial reflections in living muscle from filaments whose presence was indicated by very early electron micrographs, I soon found a clear set of reflections coming from a hexagonal array of filaments, spaced out about 400Å apart (Huxley, 1951). Moreover, in muscles in rigor, a second set of filaments seemed to be present, arranged in a regular pattern symmetrically between each set of three of the original filaments. Since it was known then that the muscle proteins actin and myosin seemed to form some kind of complex in the absence of ATP (the condition in a rigor muscle), and to dissociate in its presence, I concluded – an inspired guess, I suppose – that myosin and actin must be present in separate, stable filaments in muscle, the myosin filaments forming a permanent regular hexagonal array, and the actin filaments becoming regularly positioned in the array when they became attached by cross-linkages to the myosin filaments, who centers would be about 250Å away from those of the actin filaments. I presumed that it was interaction through those cross-links that produced muscle contraction, but because I assumed that this double array extended continuously through each sarcomere of the muscle, I did not envisage a sliding filament mechanism at this time. Instead, I wondered if actin depolymerization might be involved, because, even then, such depolymerization was recognized as a possibly biologically important process.

Additionally, I found that there was a clear set of axial reflections (I only had a slit camera), with a period of about 415Å – only approximate because the patterns I could record were so small – and a very strong third order at around 140Å. Remarkably, when a living muscle was passively stretched, this periodicity remained constant! (Huxley, 1953a) So I speculated in my thesis that the periodicity must come from one of the sets of filaments which was not attached to the Z-lines, probably the actin filaments, using the myosin filaments to transmit the force generated by their depolymerization during contraction!
3. WORK AT M.I.T.

The next step was first to find out whether my double array model was correct and then look for further evidence of how such a system might work. The best way to do this seemed to be electron microscopy, just beginning to be used in biology in a few labs in the world, one of which was F.O. Schmitt’s lab at the Massachusetts Institute of Technology, where Dick Bear and Cecil Hall also worked. I was lucky enough to get a nice two-year fellowship (Commonwealth Fund) to do this, and arrived there in September 1952. Alan Hodge was also there as a postdoctoral fellow, from Australia, and he taught me how to operate the electron microscope. Together with Dave Spiro, we designed a simple microtome for ultra-thin sectioning (Hodge et al, 1953), just coming into use then, which Alan and Dave used for their own projects and I used to look at cross-sections of muscle, for my double array. I was very thrilled to soon find I could see it. The thicker filaments formed the basic hexagonal array, as I had supposed, and presumably contained myosin, since that was the major protein species, and the thinner filaments were actin, located at the trigonal positions of the lattice, as expected from the X-ray patterns. This convinced me that the combination of the two techniques was a very powerful tool indeed, and this became one of the main themes of the Structural Studies Division which Aaron Klug and I later directed at the much enlarged MRC Laboratory in Cambridge.

By Christmas 1952 I was ready to move on with this work, and by great good fortune, Jean Hanson, from the MRC Laboratory at King’s College London, then arrived at M.I.T., also to learn electron microscopy. One of the specialities of the King’s Lab had been different types of light microscope, including the phase-contrast light microscope. Jean was a zoologist, and had already studied a range of muscle types, but she had obtained particularly striking phase-contrast light micrographs of separated myofibrils from vertebrate striated muscle (rabbit psoas), which showed the sarcomere band pattern extremely clearly. This was a revelation to me, since I had never seen the muscle band pattern in the light microscope before, though of course I had seen it in electron micrographs. Jean was equally excited to see my EM and X-ray results, and we immediately decided to join forces, and to work together using both phase and electron microscopy.

4. MYOSIN FILAMENTS IN THE A-BAND

At that time it was generally assumed that the characteristic high density of the A-bands in striated muscle was due to the presence of some additional component other than actin and myosin. Smooth muscles contracted perfectly well, if more slowly, without such striations, and since sarcomeres could shorten down to much below the A-band length, it was assumed that filaments of the actin-myosin complex must extend continuously from one Z-line to the next. So perhaps this extra A-substance just enabled muscles to shorten faster?

We were absolutely astounded, therefore, when, in the phase contrast light microscope, we saw that myosin-extracting solutions removed the A-substance, but left behind a ‘ghost’ fibril, with a band of density on either side of each Z-line, extending in towards where the boundaries of the H-zone had originally been. (H-zone is a less-dense region in the center of the A-band). Within a day we realized what the explanation must be, and were soon able to confirm this by electron microscopy. Myosin-extracting solutions removed the thick filaments, leaving behind the sets of thin filaments (attached to the Z-lines), which had previously partially overlapped the thick filaments. When I had seen the double array in an end-on view, I had been looking at sections through the overlap region of the sarcomere, and what I thought was poor preservation or sectioning were just the I-regions!
EARLY DEVELOPMENTS IN MUSCLE RESEARCH

This was a remarkable finding, which we published in Nature that year (Hanson & Huxley, 1953), though it took a long time to come out. We were told by Schmitt that we should not contaminate a perfectly good experimental paper with any speculation about mechanisms until we had further evidence. However, I did manage to slip some phrases to the effect that my constant X-ray axial periodicity, plus the overlapping arrays, suggested a sliding mechanism, into my write-up of the electron microscope cross-sectioning results (Huxley 1953b).

By January of 1954 we had good phase microscope data showing that the A-bands remained essentially constant in length during contraction of isolated myofibrils in ATP, and that the arrays of thin filaments, also of constant length, were drawn further and further into the A-bands as contraction proceeded. And so the sliding filament model was published in Nature in May of 1954, in two papers side by side, one from us, and one from A.F. Huxley and Niedergerke, who had been making similar observations on intact muscle fibers in the interference light microscope (Huxley & Hanson, 1954; A.F. Huxley and Niedergerke, 1954).

Subsequent development of the work took place in England again, and continued to be very dependent on new technical developments.

5. DEVELOPMENTS IN ELECTRON MICROSCOPY

Some of these were fairly straightforward. Thin sectioning for electron microscopy was still a very new technique, and I only slowly realized that most sections were over a thousand angstroms in thickness, and relatively lightly stained, so that, in longitudinal sections of muscle, several layers of filaments were superposed, obscuring the filament arrangement. By various incremental improvements of the microtome and of specimen preparations, I managed to reduce the minimum section thickness to 100-150Å, and to have sufficiently intense metal staining for single filaments to show up with good contrast. Remarkably, the double hexagonal lattice was often preserved with great regularity, so that single filament layers could be seen with thick and thin filaments lying side by side in the expected arrangement, within the thickness of the section. This began to persuade people that the overlapping filament structure really did exist. But the sliding filament mechanism still took many years of work to gain more converts.

I was disappointed that the staining methods that I had developed for thin sections showed little internal detail of the structure of the actin and myosin filaments, although it was possible to see crossbridges very clearly. However, I had accidentally discovered the so-called ‘negative staining’ technique in some work I was doing on tobacco mosaic virus, as a sideline (Huxley, 1957b), and I improved it further by using uranyl acetate as a negative stain, in work on another virus, and on ribosomes (Huxley & Zubay, 1960a & 1960b). The technique could only be used on small isolated objects, which were submerged or outlined in the stain, but it occurred to me that muscle might fragment easily if mechanically blended in a relaxing medium, and that turned out to be the case. The product was a nice suspension of separated and often unbroken myosin and actin filaments, which showed up excellently in negative stain (Huxley, 1963). This enabled me to recognize that both types of filament were constructed with a defined structural polarity, appropriate for the directions in which relative sliding forces needed to be developed in an overlapping filament system. This was further strong evidence in support of such a mechanism, and it also prompted me to suggest that similar directed movements might be involved in other forms of cell motility. It also enabled Jean Hanson and Jack Lowy (1963) to elucidate the helical structure of F-actin.
6. X-RAY DEVELOPMENTS IN THE 1960'S

The major developments came next from the X-ray field. I was now back in the new MRC laboratory in Cambridge and had access again to rotating anode X-ray tubes, several of which were operating routinely there. Ken Holmes and Bill Longley had also moved to the laboratory from Birkbeck College, in London, and had made a big improvement to such a tube by grafting on the cathode from a Boudoin X-ray tube, which provided a much smaller focus and hence light brilliance, very appropriate for the focusing quartz monochromator they were using in studies on Tobacco Mosaic Virus. Ken and I thought it would be interesting to combine this with a focusing mirror I had previously been using in conjunction with a commercial version of the Ehrenberg-Spear tube. In the course of playing around with the rotating anode-mirror-monochromator set up, I discovered that it was possible to use the entire input aperture of the monochromator, rather than a narrow collimated region (as was conventional), without excessive background scattering, with an enormous increase in total X-ray intensity and all the advantages of a monochromatic beam.

This made it possible for the first time to record the low angle meridional and layer-line patterns from live, contracting frog muscles, to show that the axial spacings from both myosin and actin remained essentially constant between rest and contraction (Huxley, Brown & Holmes, 1965), and to show, amongst other things, that the myosin layer line reflections from the helical arrangement of crossbridges around the myosin filaments, became very much fainter during contraction, showing that the crossbridges must move when they developed the sliding force between the filaments (Huxley and Brown, 1967).

Holmes and I were able to continue to improve the technology because of the excellent workshop facilities and technicians in the lab. In a successful effort to achieve higher X-ray output, we constructed a large diameter (about 20 ins) rotating anode X-ray set, which eventually developed into the commercial Elliott 'Big Wheel'. But we realized that this was essentially the end of the line as far as increases X-ray intensity from these types of sources was concerned, since we had reached the limit of what the mechanical strength and the melting point of copper would allow, and other metals would give X-rays with a much less suitable wavelength, and present much bigger fabrication problems. Nevertheless, we still needed greatly increases X-ray fluxes, since there was potentially so much detailed information available in the diagrams from contracting muscle, and we needed to be able to record them with high time resolution to follow the changing patterns satisfactorily.

7. SYNCHROTRON RADIATION

Ken Holmes had always been thinking about more exotic X-ray sources, particularly for use in his work on insect flight muscle, and had become interested in the radiation emitted by electron synchrotrons, particle accelerators used by physicists to produce very high velocity electrons for collision experiments. Initially, it appeared that the available machines would not produce enough X-rays to be useful sources, but later, after he had moved to Heidelberg, and had learned from Gerd Rosenbaum about the characteristics of the DESY synchrotron in Hamburg, things appeared more hopeful, and he, Rosenbaum, and Witz carried out the crucial test experiment there in 1971 (Nature, Rosenbaum, Holmes, & Witz, 1971). This showed that a substantial gain was available over the best that could ever be obtained from a rotating anode X-ray tube, and that potentially enormous factors of improvement might be possible in the future from the electron storage rings being planned, in which much larger, and continuous, circulating currents of electrons and positrons would be present.
In any event, it took nearly ten years more before all the component of a satisfactory system were available. It took some years before a storage ring came on line, and was operating smoothly enough (not very smoothly!) for the muscle enthusiasts to begin to collect some really useful data. In the meantime, we gained a lot of essential experience with electronic data collection and analysis, and with the operation of remotely controlled cameras, at the synchrotron NINA, in Daresbury, U.K. (because of the high flux of radiation in the immediate environment of such a beamline, remote operation was mandatory). Once again, in the MRC lab, we were very fortunate that nearly all the equipment could be designed by excellent electronics and mechanical engineers and put together in our workshops (Haselgrove et al, 1977; Faruqi & Bond, 1980).

And then, in the early 1980s, working at the EMBL Outstation, in Hamburg (specially built to exploit synchrotron radiation), we were able to obtain good time-resolved patterns of the myosin and actin layer-lines with 5-10 msec time resolution, so that the time course of the changes in them during the onset of contraction could be compared with the time course of tension development. They were very closely related as we expected. More importantly, in measurements of the meridional 145Å reflection from the myosin crossbridge repeat, now with 1 msec time resolution, we found that a large drop in intensity was closely synchronized with a small quick length decrease, or increase, applied to a previously isometrically contracting muscle. This strongly indicated a tilting movement of actin-attached crossbridges, if they were approximately perpendicularly oriented in the isometric muscle, and provided the first direct demonstration of a direct relationship between crossbridge configuration and tension generation (Huxley et al 1981, 1983).

8. RECENT ADVANCES

Later development of new structural techniques in other laboratories have led to further remarkable extensions of our ability to obtain direct information about molecular motility processes. Perhaps the most striking of these has been the introduction of in vitro motility measurements in which force and movement can be measured by direct optical means on single myosin molecules interacting with single actin filaments, as exemplified, for instance, by Finer et al (1994), following the pioneering work of Kron and Spudich (1986) and Kishino and Yanagida (1988).

Another great step forward depended on extensive computerization of protein crystallographic analysis, to make the solution of very large structures possible, plus the use of synchrotron radiation and cryo techniques to collect data from very small and sensitive protein crystals. This made possible the solution of the high resolution structure of myosin subfragment one, the motor part of myosin, by Rayment et al. (1983a & 1983b), a very great help to our understanding of the details of the tilting movement of myosin crossbridges in the sliding filament mechanism.

More recently still, the latest generation of electron-positron storage rings provide extremely small X-ray sources with very high total flux. These make it possible to record X-ray patterns from muscle at extremely high spatial resolution and at a time resolution of 1 msec or better, making use of imaging plates and improved CCD X-ray detectors (Linari et al, 2000; Huxley et al, 2000). Such patterns enable axial crossbridge movements to be measured with an accuracy of a few angstrom units.

9. CONCLUSION

Over the last fifty years or so, then, it has been very remarkable, and encouraging, to see how the well-directed scientific efforts of many people and many
laboratories have been so successful in providing the tools for what were originally almost unimaginable opportunities to explore molecular reality.

REFERENCES


EARLY DEVELOPMENTS IN MUSCLE RESEARCH


DISCUSSION

Gonzalez-Serratos: In your early experiments with Jean Hansen, you showed that, after extraction of myosin when only actin was left, the sarcomere decreased in length. How could they shorten without myosin?

Huxley: No. In the experiments I showed, myosin had been extracted after the contraction had taken place, so as to show more clearly the location of the I-segments in the shortened sarcomeres.

Pollack: In one of your EM slides showing two thin filaments between two thick filaments, we can see two kinds of bridges: between thick and thin and between thin and thin. We found the thin-thin connections even in the I-band (cf. Pollack, Muscles and Molecules, Ebner & Sons, Seattle, 1990). How do you interpret the thin-thin bridges that you see in the EM, and why do they also appear in the I-band?

Huxley: In the I-band, the thin-thin bridges may be some of the glycolytic enzymes which bind quite strongly to actin. In the A-band, they may also represent myosin crossbridges from myosin filaments above and below the actual plane of sectioning.
THE MOLECULAR BASIS OF CROSS-BRIDGE FUNCTION

Kenneth C. Holmes

1. INTRODUCTION

Our understanding of the physiology of muscle depends critically on the resolution of the available anatomy. Early insight was provided by light microscopy. However, the first radical new insight was provided by electron microscopy. Ultimately, an understanding in physicochemical terms is only possible if the structures of the components in various physiological states are known at atomic resolution. Some of these have become known in the last 15 years and now allow us to describe how the hydrolysis of ATP by the component proteins actin and myosin leads to movement.

HE Huxley was able to show that the filaments in the sarcomere were organised on a hexagonal lattice \(^1,2\). The seminal works of HE Huxley and Jean Hanson \(^3\) and AF Huxley and Niedergerke \(^4\) showed that the two sets of filaments in the sarcomere glide over each other without altering their length. Through the work of HE Huxley, Jean Hanson and W. Hasselbach it was discovered that the thick filaments contain myosin and the thin filaments contain actin. The myosin molecule consists of two heavy chains and four light chains. A soluble proteolytic fragment of myosin, heavy mero-myosin that contains the 2 globular "heads" of myosin carries the ATP-ase activity \(^5\), the rest of the molecule forming a long double \(\alpha\)-helical coiled-coil involved in filament formation. The ATP-ase activity was later shown to reside in the "head" fragment (sub-fragment-1 or S1)\(^6,8\) that constitutes the cross-bridge (see below).

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2. CROSS BRIDGE THEORIES

2.1 The swinging cross-bridge

After the discovery of the sliding filaments the question naturally arose; what made them slide? AF Huxley argued that the source of the force must be independent elemental force generators since the force increases linearly with the degree of filament overlap. The myosin cross bridges were first visualised by electron microscopy and subsequently shown both to be the site of the ATP-ase and also to be the motor elements producing force and movement between the filaments. Two conformations of the cross-bridge could be detected in insect flight muscle by low angle X-ray scattering and electron microscopy. The cross bridge attaches to the actin filament at about 45° in rigor and at right angles (90°) in the presence of ATP.

On the basis of the known structural data and their kinetic analysis Lymn and Taylor proposed the cross-bridge cycle (Fig. 1). The cross bridge was thought work by a kind of rowing action. Initially it would bind to actin in a 90° conformation, go over to an angled (45°) conformation and then release the products of hydrolysis. The rebinding of Mg-ATP rapidly dissociates the actin-myosin; myosin then hydrolyzes ATP and forms a stable myosin-products complex; actin recombines with this complex and dissociates the products thereby forming the initial actin-myosin complex that isomerises to the rigor complex. Force is generated during the last step.

The actual rowing movement could be measured by physiological experiments on contracting muscle and was shown to be about 100Å. Since the cross-bridge was an elongated structure, such a distance could be accommodated by a rotating or swinging cross-bridge model (Fig 1a). Studies of the cross-bridge movement were undertaken by time resolved studies of contracting frog muscle using low angle x-ray fibre diffraction. These results are fully consistent with the swinging cross-bridge theory.

2.2 Swinging lever arm

Although the swinging cross bridge hypothesis of muscle contraction had become the textbook norm by the time of the Cold Spring Harbor Conference on Muscle in 1972 it proved remarkably difficult to catch a bridge in flagranti delicto (Cooke).
Nevertheless, the swinging cross-bridge hypothesis provides by far the best framework for correlating and explaining the large muscle literature. The hypothesis has been modified over the years into a *swinging lever arm hypothesis* in which the bulk of the cross bridge is envisaged to bind to actin with a more or less fixed geometry and only the distal (C-terminal) part of the myosin molecule moves (Fig. 1b). A swinging lever arm explains why substantial changes in the cross bridge orientation were difficult to detect: only a small fraction of the cross-bridge mass moves. Furthermore, it became clear that the proportion of cross bridges in a muscle fibre taking part in a contraction was at any one time only a small fraction of the total, making the registration of active cross bridge movement doubly difficult.

The atomic structures of actin and myosin provided new impetus. The crystal structure of the myosin subfragment 1 showed the myosin cross-bridge to have an extended C-terminal tail which looked like a lever arm and, moreover, a lever arm which was in the correct orientation and position to function as a lever arm. In the last years large numbers of independent experiments provide results, which are in excellent accord with the idea that the C-terminal tail functions as a lever arm and indeed provide evidence that it can move. Purified myosin cross bridges (S1) can be attached to a substrate and used to transport actin filaments in vitro in the presence of ATP. A study by Spudich *et al* showed that the speed of actin transport in motility assays was proportional to the length of the lever arm. This experiment has
now been repeated a number of times on various myosins. Single molecule measurements come to similar conclusions. The bulk of the published data agree very well with the swinging lever arm model.

In addition, new crystal structures showed that the myosin cross bridge can exist in two orientations corresponding to the two end states of the power stroke.

3. PROTEIN CRYSTALLOGRAPHY

3.1 Structure of Actin

Actin (thin filament) fibres are helical polymers of g-actin (globular-actin) 22. The structure of g-actin was first solved by protein crystallography as a 1:1 complex with the enzyme DNase I 23. Orientated gels of f-actin yield X-ray fibre diagrams to about 6Å resolution. Holmes et al determined the orientation of the g-actin monomer that best accounted for the f-actin fibre diagram and thus arrive at first approximation to the atomic model of the actin filament 24. Since a conformational change is involved in going from g- to f-actin, the g-actin structure has to be deformed in some way to fit the f-actin diffraction pattern. The various attempts at generating the f-actin structure that have been published have chosen various methods of defining these free parameters and have ended up with related, but different, solutions to the problem 24-26. The method of Lorenz et al 26 produces a very good fit to the fibre diffraction pattern but with a large number of free parameters and at the cost of poor stereochemistry. A new attempt to solve this problem has been made that is based on the sub-domain structure of actin and a small number of degrees of freedom 27. The g-actin structure from Otterbein et al 28 was used as the starting structure.

3.2 Structure of Myosin

The cross-bridges comprise a part of the myosin molecule, namely subfragment-1 of heavy meromyosin (S1). X-ray crystallography 19 shows the chicken skeletal S1 to be tadpole-like in form (fig. 2) , with an elongated head, containing a 7-stranded β-sheet and numerous associated α-helices forming a deep cleft. The cleft separates two parts of the molecule, which are referred to as the upper 50K, and lower 50K domains. The C-terminal tail, sometimes called the "neck", which also provides the connection to the thick filament, forms an extended α-helix that binds two calmodulin-like light chains. The ATP binding site consists of a "P-loop" motive flanked by switch 1 and switch 2 elements, as are found in the G-proteins.

By fitting the atomic structures of f-actin and S1 into three dimensional cryo-electron microscope reconstructions one arrives at an atomic model of the actin myosin complex 20 (fig. 3). In particular, this model establishes the spatial orientation of the S1 myosin fragment in the active complex. One finds that the cleft in myosin extends from the ATP binding site to the actin binding site and that
the opening and closing of this cleft is very likely to provide the communication between the ATP site and the actin binding site. The actin-binding site spans the upper and lower 50K domains. Furthermore, the very extended C-terminal α-helical neck of S1 is ideally placed to be a lever arm. The lever arm joins onto the bulk of the molecule via a small compact "converter domain" \(^{29}\) which lies just distal to a broken α-helix containing two reactive thiol groups known as SH1 and SH2. Numerous experiments point to the putative "hinge" for the lever arm being in the SH1-SH2 region of the molecule (see \(^{30}\) for review). The converter domain is the socket that carried the C-terminal helix (lever arm).

Figure 2 The structure of the myosin cross-bridge as a ribbon diagram\(^9\). The N-terminus is shown green and the nucleotide binding P-loop is shown yellow. The strut (yellow) connects the upper and lower 50K domains. Note the cleft separating the upper and lower 50k domains. The lower 50K domain is the primary actin-binding site. The upper and lower 50K domains are also connected by a disordered loop (not shown). The SH1 SH3 region lies underneath the right part of the relay helix. The C-terminal long helix (dark blue) carries two calmodulin-like light chains and joins on to the thick filament.

3.3 Two main conformations of myosin have been discovered

According to the Lymn-Taylor scheme (fig. 1) the myosin cross bridge would be expected to have two discernible conformations: (1) when it first attaches to actin with the products of hydrolysis still bound with the lever at the beginning of the working stroke; and (2) at the end of the working stroke when the phosphate and ADP are released. This sequence is often referred to as the "power stroke". The end state is referred to as "rigor", since it is the state muscle enters on ATP depletion. It is also called "strong" because it binds to actin quite tightly. The initial state is called the "weak binding state" because of its low affinity for actin (see \(^{31}\)), although it may be necessary to specify the pre-power stroke bound state as stereo-
specific weak binding. We might anticipate that these two states of the myosin cross bridge might exist independently from actin and indeed protein crystallography shows this to be the case. The cross-bridge exists in two main conformations, the lever arm undergoes a 60° rotation between these two states, which have been identified as the beginning and end of the power stroke. The lever arm rotation is coupled with changes in the active site (the movement of the switch 2 element from closed to open) and to product release.

Figure 3 The pre (upper) and post (lower) power stroke states of the myosin cross bridge, as they would appear when attached to actin. The actin filament is shown on the right. These two states are referred to as CLOSED (upper) and OPEN (lower) because in the CLOSED state the switch 2 element has move in to close off the active site. This state is the ATP-ase. Note the large movement of the distal lever arm, which undergoes a 60° rotation between these two states,
3.3.1 The OPEN state (post power stroke)

The first chicken S1 structure was solved without bound nucleotide. The chicken S1 crystal structure fits well into the electron micrograph reconstructions of the strong actin-myosin nucleotide-free interaction (decorated actin). Therefore the crystal structure of chicken S1 would appear to represent the end of the power stroke in a near rigor state. The switch 2 element lies away from the nucleotide binding pocket. Hence we refer to this state as the OPEN state. It has been found in the presence of a large variety of ATP analogues.

3.3.2 The CLOSED state (pre-power stroke)

Rayment et al have extensively studied a crystalline fragment of the dictyostelium myosin II cross-bridge which has been truncated after residue 761 (equivalent to 781 in chicken skeletal sequence). The truncation eliminates the lever arm and the associated light chains but retains the converter domain. The crystal structures of the 761 construct have been determined with a number of ATP analogs, particularly ADP.BeF₃ and ADP.vanadate. ADP.vanadate complexes are used as analogs of the transition state or possibly of the ADP.Pi state. While the ADP.BeF₃ state looks similar to rigor, the ADP.vanadate structure shows dramatic changes in shape of the S1 structure. There is a closing of the γ-phosphate binding pocket by moving switch 2 element about 5Å. This induces large movements in the C-terminal region of the molecule. The converter domain rotates through 60°. This new state has been called “CLOSED” (because the nucleotide pocket is closed) or “transition state” because it seems to be produced by transition state analogues of ATP.

Dominguez et al have solved the structures of chicken smooth-muscle myosin truncated at 791 (smooth muscle sequence) or at 820 (expressed in insect cells using the baculovirus vector). The shorter construct stops at the end of the converter domain and the longer construct encompasses the essential light-chain binding site that is, the first half of the lever arm. The structures of both constructs have been solved as complexes with ADP.vanadate and ADP.BeF₃. Both structures show the myosin cross-bridge in the CLOSED form with the converter domain in the rotated position very similar to that obtained in the two Dictyostelium constructs. The authors refer to this state as the “pre-power stroke state”. There are no substantial differences between ADP.AlF₄ complexes and ADP.BeF₃ complexes showing that the nature of the ligand (either a transition state analog or an ATP analog) does not control the protein conformation very closely. Since the smooth-muscle crystals display extensive non-crystallographic symmetry, CLOSED has now been obtained 16 times in a large variety of different environments: there is little chance that it arises as an artefact of crystal packing.

The two states are depicted in fig. 3 as they would appear if they were full length cross bridges attached to actin. (The missing lever arm has been added in). The lever arm moves some 12nm along the actin helix axis between the two states.
The movement of the lever arm and the status of the nucleotide-binding pocket are tightly coupled: pocket closed, lever up (beginning); pocket open, lever down (end). Only the CLOSED form is an ATPase. This is an essential control to make the cross-bridge cycle efficient.

Figure 4 (Upper) Pre-power stroke and (lower) post-power stroke (near rigor) states. The removal of the bulge in the relay helix drives the rotation of the converter domain. The breaking of the relay helix appears to result from the helix being pressed against the β-sheet when the switch 2 element moves so as to hydrogen bond with the γ-phosphate. The colour coding is the same as in Fig. 2.
3.3.3 the relay helix bulge is the quintessence of muscle contraction

In the CLOSED state the upper/lower 50K domains rotate a few degrees with respect to each other so as to bring the invariant gly457 (466 in chicken skeletal myosin) amide group into H-bond contact with the g-phosphate - a movement of 5Å. At the same time the long relay helix, (residues 475-507 in *dictyosteleum*) bends and breaks to form a helix bulge (i.e. skips one hydrogen bond), which causes a rotation of the distal end of the helix and a rotation of the attached converter domain (711-781) by 60°. The relay helix breaks because it is forced into close contact with the β-sheet by the moving in of the switch 2 element. The fulcrum for the rotation of the converter domain is provided by the mutual rotation of the distal part of the SH1-SH2 helix around the distal part of the switch 2 helix. The relaxation of this bulge in the relay helix is the essential event that drives the lever arm down. It can come about through the moving out of switch 2 from the actin site on release of the g-phosphate. It can also come about by a twisting of the backbone β-sheet of the cross bridge. This second possibility has been shown by two recent structures of the myosin cross-bridge[^35][^36].

4. STRUCTURAL EVIDENCE FOR MOVEMENT OF THE LEVER ARM IN THE ACTIN-MYOSIN COMPLEX

Whereas the structure of "decorated actin" an actin filament with a myosin cross-bridge bound to each actin in the rigor state has been extensively studied[^37]-[^40] corresponding studies in the presence of ATP are difficult since the binding of ATP leads to rapid dissociation of the cross-bridges from actin. Time-resolved electron micrograph studies in fact show no bulk change of the cross bridge orientation on binding ATP before dissociation takes place[^41] whereby a reorientation of the lever arm would not have been detected at the resolution attainable. High-resolution electron micrographs of actin decorated with smooth muscle myosin, however, show a 30-35° rotation of the lever arm on binding ADP[^42][^43]. Although the main movement of the lever arm would be expected to be associated with phosphate release since this is a step associated with a large change in free energy, some fraction of the movement could arise from ADP binding and release. Moreover, this movement should be recoverable on adding ADP to actomyosin, which indeed it is. Although the effect has only been found in smooth muscle myosins this experiment was important in providing the first direct demonstration of a nucleotide-induced lever-arm swing.

5. MYOSIN V, A PROCESSIVE MYOSIN

Myosin II and myosin V have rather similar structures except that the lever arm on myosin V is long enough to binds 6 calmodulin light chains rather than 2 (myosin II cross bridges are about 16 nm long, myosin V cross bridges about 31 nm long). Whereas the two heads of myosin II act independently the two heads of
myosin V bind conjointly to the actin filament to produce a processive linear motion along actin by a hand over hand mechanism. The myosin V lever arms are long enough to allow two-headed binding that spans the actin helical repeat (approximately 36 nm). Electron microscopy has shown that while working, myosin V spans the actin helical repeat. The heads are mostly 13 actin subunits apart. Typically the lead head appears curved. The leading head may correspond to the beginning of the working stroke of the motor. Besides providing a graphical demonstration of the swinging lever arm myosin V offers the possibility of studying the elusive top-of-power-stroke state.

7. WEAK AND STRONG BINDING

The actin binding site straddles a cleft between two sub-domains, the upper and lower 50K domains. Kinetic analysis shows that the initial binding of myosin to actin is "weak" and that weak binding isomerises to "strong." Strong binding drives the power stroke and affects ATP and ADP affinity. All myosin II crystal structures whether OPEN or CLOSED have an open 50K domain cleft and appear to be in the weak actin binding form. Since myosin V binds more tightly to actin it there is a chance that the myosin cross-bridge alone might be found in the strong binding form. X-ray crystallography has recently given the structure of the myosin V cross-bridge and indeed it appears to be in the strong binding form. Moreover, the interaction between the myosin cross-bridge and actin can be studied by cryo-electron microscopy. Together these studies explain the reciprocal linkage between actin binding and nucleotide binding: on strong binding to actin the 50k domain cleft closes leading to a movement of the switch 1 element out of the active site; the rebinding of ATP closes the active site by pulling in switch 1 thereby opening the cleft and weakening actin binding.

8. CONCLUSION

Three of the four states anticipated by the Lymn-Taylor cross-bridge cycle are now known in atomic detail. The "unbound" states, which are more easily available to protein crystallographic analysis, have yielded most information. The connections between ATP binding and the conformation of the myosin cross-bridge in solution are well understood. Besides being an ATPase the myosin "head" has two essential functions: a shape change induced by product release that drives contraction; a large change of affinity for actin induced by binding ATP. X-ray crystallography, in conjunction with electron microscopy has recently yielded an explanation of both these phenomena in molecular terms. The detailed structures of the actin bound states are gradually becoming available by combining crystal structures analysis with high-resolution electron microscopy. However, the structure of the ephemeral strong binding at the beginning of the power stroke can at present only be inferred.