Muscle contraction is produced by the intermittent and asynchronous “working strokes” of many individual myosin molecules that “row” the “thin” actin filaments past the “thick” myosin filaments. Actomyosin cross bridges are formed by the “heads” of the myosin II molecules that protrude from the shaft of the myosin thick filament and interact with actin filaments in a cyclical manner, hydrolyzing a single ATP molecule in each cycle. Muscle myosin II molecules are nonprocessive molecular motors, i.e., ones that are released from actin after each catalytic cycle. In contrast, some nonmuscle (or “unconventional”) myosins are processive motors that undergo multiple catalytic cycles and mechanical steps for each diffusional encounter with actin. The myosin superfamilies consist of at least 18 different classes distributed across plant and animal kingdoms and with great diversity of cellular functions (17). Reflecting these functional differences, there is considerable sequence and structural diversity in the tail part of the molecule, whereas the motor domain or head of the molecule is well conserved. It is assumed that throughout the myosin family the basic mechanism of movement and force production is the same and occurs, as already mentioned, by the cyclical interaction of the myosin motor domain with F-actin coupled to the breakdown of ATP.

Of all of the different members of the myosin family, muscle myosin II is surely the best studied. It provides a paradigm for studying the structure/function relationships and how conformational changes might generate movement and force. Here we will focus mainly on our current understanding of this myosin that has been optimized for a variety of contractile functions, from the rapid repetitive contraction cycles of insect flight muscles to the extremely slow contractions of vertebrate smooth muscle. In addition, we will also touch on some single-molecule studies with unconventional myosin I. The recent discovery of a two-step working stroke of myosin I gives important insight into the mechanism of cross-bridge movement and force production. These experiments (described in Ref. 19) might offer insight into the molecular mechanism that allows force maintenance to be economical from an energetic point of view, in particular in a state of tonic contraction such as “latch” and “catch” of vertebrate and invertebrate smooth muscles. Recently developed single-molecule mechanical techniques have made it possible to address the mechanism of movement and force production during a single cross-bridge cycle in an unequivocal way.

Structure of myosin II motors

Muscle myosin II heads [proteolytic subfragment 1 (S1)] consist of 1) an NH2-terminal catalytic (or motor) domain containing the actin-binding sites and the ATPase catalytic site and 2) a neck region formed by an extended α-helix, to which are bound the essential and regulatory light chains (Refs. 7 and 15; cf. Fig. 1). S1 retains all of the motor functions of myosin in vitro, i.e., the ability to produce motility and force. Furthermore, limited proteolysis breaks S1 into three fragments named after their apparent molecular weights: 25 kDa (NH2-terminal), 50 kDa (middle), and 20 kDa (COOH-terminal). The crystal structure of S1 shows that these proteolytic fragments, rather than representing subdomains of S1, mark the positions of two flexible loops (loop 1 and loop 2). It transpires that these loops are highly variable among myosin classes and are important in determining the biochemical and perhaps mechanical properties of different myosins.

A long α-helix, stabilized by the two noncovalently bound light chains, projects from the ATPase catalytic site toward the COOH-terminal tail of the molecule. It has been suggested that this “regulatory domain” (neck domain) acts as a “leaver arm” structure that plays a central role in the production of mechanical force and movement during the working stroke of the cross bridge. To date, several crystallographic structures of chicken and scallop fast skeletal muscle myosin and vertebrate smooth muscle myosin with various nucleotide analogs bound in the nucleotide-binding pocket have been solved. On the basis of these crystal structures, it has been hypothesized that the cross-bridge working stroke is produced by an angular movement of the myosin regulatory domain about a fulcrum in the so-called converter region of the myosin head. The regulatory domain therefore acts as a lever to amplify small structural changes that take place close to the catalytic site in the so-called switch I and II regions (Refs. 1 and 7; cf Fig. 2).
Subnanometer rearrangements at the active site are geared up to give a displacement of ~5–10 nm at the end of the lever arm. This lever movement would then drive a displacement of the actin filament relative to the myosin head, and by deforming internal elastic structures it would also produce force (8). Part of that movement has been seen in cryoelectron microscopy studies of smooth muscle myosin bound to actin (20). An overall displacement of ~10 nm was estimated from X-ray crystallographic studies of the motor domain of smooth muscle subfragment 1 crystallized in the presence and absence of ATP analogs (5). However, since crystal structures of the actomyosin complex are still elusive, we do not know the effect of actin binding to myosin on its head structure in general and, specifically, on the conformational change of the lever arm occurring during the working stroke of cycling cross bridges.

The cross-bridge cycle

During each cyclical interaction of myosin with actin, one molecule of ATP is hydrolyzed by the myosin head into ADP and inorganic phosphate (Pi). Myosin binds to actin with the products of hydrolysis bound in the catalytic site to form an actomyosin-ADP-Pi complex (cross-bridge attachment). The subsequent, actin-activated release of Pi and then ADP is associated with a conformational change of the myosin head—the working stroke—that generates movement. Binding of a new ATP molecule to myosin causes the actomyosin rigor complex to dissociate (cross-bridge detachment), and subsequent ATP hydrolysis resets the original myosin conformation (“recovery stroke”) for another biochemical and mechanical cycle. The rowing action of cross bridges requires that the myosin affinity for actin is strong during the working stroke but weak during the recovery stroke.

Single-molecule mechanics

Development of single-molecule techniques over the past 10 years have made it possible to study force and movement produced by myosins in great detail. Most single-molecule mechanical studies of actomyosin use an optical tweezers-based transducer. The apparatus is usually built around a fluorescence light microscope and uses near-infrared laser light to produce the optical tweezers. Finer and colleagues (6) used this method to measure the piconewton forces and nanome-
ter displacements produced by single actomyosin interactions. In these experiments, small numbers of myosin molecules are deposited on a solid support, e.g., a small (~2 μm diameter) silica bead, fixed to the surface of the microscope coverglass. A single myosin head is then brought into the vicinity of an actin filament, bound at either end to a plastic bead (~1 μm diameter) that is held and positioned by laser traps (see Fig. 3). The position of these plastic beads is monitored by projecting their images onto a photodetector. The data trace in Fig. 3 shows the movement of one of the plastic beads parallel to the actin filament axis during such an experiment. The bead-actin-dumbbell exhibits pronounced thermal motion that is occasionally interrupted by intervals of reduced thermal motion. It is now generally agreed that intervals of “high noise” represent periods of Brownian motion when myosin is not attached to the actin filament, whereas “low-noise” intervals identify periods of cross-bridge attachment to actin (13).

In the presence of ATP, single myosin heads attach to actin, perform a working stroke, and then detach after a stochastically distributed time interval that is dependent on the ATP concentration in solution. Some aspects of these experiments are analogous to single-channel recordings using patch clamp methods, in which the kinetics and conductivity of a channel type can be determined from ensemble averaging of channel opening events. Both the kinetics and the size of the working stroke of a cross bridge can be determined from the single-molecule mechanical data. Using ensemble averaging and so-called forced oscillations to improve time resolution to ~1 ms, the working stroke of rabbit fast skeletal muscle myosin II was measured to be ~5 nm and was completed within 5 ms (Fig. 4, bottom).

Using rather different approaches for measurement and data analysis, most groups have confirmed a ~5 nm working stroke for mammalian fast skeletal myosin S1 (e.g., Refs. 6, 13, 18, and 19), whereas a 10- to 12-nm working stroke was estimated from mechanical studies of single muscle fibers (cf. Ref. 8). In contrast to these findings, Yanagida’s group has claimed that attached cross bridges may ratchet along actin sites in a series of up to seven steps, slipping along the actin filaments over 5–30 nm for each cross-bridge encounter (12), thus questioning the lever arm hypothesis altogether.

**The lever arm hypothesis of the cross-bridge working stroke**

If the lever arm hypothesis is correct, then the working stroke size should depend linearly on lever length. Recent studies in which the length of “engineered” lever arms has been altered (16) are consistent with the idea that the regulatory domain (neck domain) functions as a lever arm that tilts during the working stroke relative to the motor domain (and the actin filament). But these experiments also suggest that the functional length of the lever arm may be somewhat longer than the length of the neck domain. Thus comparing the step size produced by myosin constructs with shortened or elongated artificial neck domains and using the linear relationship between displacement and lever arm length, extrapolation to zero step size placed the origin of the lever arm rotation >2 nm inside the motor domain (16).

Furthermore, tomographical reconstructions of electron micrographs of insect flight muscle that had been quickly frozen during contraction suggest that the working stroke is composed of a combination of tilting (rotation) of the motor domain on actin and lever arm swinging through ~35°. Also, a surprisingly large working stroke was found in single-molecule mechanical studies with proteolytically shortened rabbit skeletal S1 (14). All of these results are in agreement with the idea that, in addition to the lever arm motion, part of the working stroke might be caused by a rotation of the motor domain about its contact with actin, as originally proposed by H. E. Huxley, A. F. Huxley, and R. Simmons (see Ref. 8 for review).

One of the current challenges in single-molecule mechanical experiments is to resolve subtle details of the separate mechanical events that might occur during the early phases of the cross-bridge attachment, specifically during P₂ and ADP release. So far, the time resolution has been insufficient to provide insight into the phase between muscle myosin II attachment to actin and the end of the working stroke. So it remains unclear whether the working stroke of the lever arm of fast skeletal muscle myosin is produced in a single step or in two or more discrete substeps, as previously suggested (see Ref. 8 for review). The experimental conditions are more favorable, however, if extremely slow myosins, specifically myosins with...
slow kinetics of ADP release, are used. Indeed, recent studies with slow, unconventional myosin I, which is a single-headed myosin, give insight into the details of this mechanism.

**Myosin I produces its working stroke in two steps**

Single-headed brush border myosin I from the microvilli of intestinal epithelia and the 130 kDa myosin I from liver are slow myosins in terms of their ATPase kinetics and in vitro motility velocities (3). The slow kinetics and the long neck length of class I myosins with between three and six light chain binding domains make them ideal for investigating the timing of the working stroke. Using these myosins, it was found that the actin filament was displaced initially by ~6 nm (within a few milliseconds of myosin binding) and then, following a time delay of 100–300 ms, a further movement of ~5 nm occurred (Fig. 4, top and middle). The time interval between the two substeps was independent of ATP concentration, whereas the lifetime of the interaction following the second substep was shortened by increasing the ATP concentration. This suggested that the phase following the second substep was terminated by ATP binding. Furthermore, the size of the second substep (~5 nm) was similar to the tilting of the light chain domain of brush border myosin I induced by the release of bound ADP, deduced from cryoelectron microscopy of myosin I-decorated actin filaments (10). It is tempting to speculate that the two substeps of the brush-border myosin working stroke are coupled to transitions between enzymatic states, such as P, release from the active site, followed by ADP release. If so, the intermediate attached state, lasting from the end of the first substep to the start of the second substep, probably corresponds to a long-lived actomyosin-ADP state. Figuratively speaking, it probably represents a “caught” state of the lever arm, which, after performing the first phase of its working stroke, may be blocked for a while before completing its movement and detaching from the actin filament.

In view of these results, the question arises as to whether a two-step lever arm mechanism and the existence of long-lived, attached actomyosin-ADP states can also be demonstrated with muscle myosin II. However, no tilting was observed in cryoelectron microscopy reconstruction studies of skeletal muscle myosin subfragment 1 in response to ADP (see review in Ref. 7), and mechanical substeps were not seen in single-molecule studies on skeletal muscle S1. One might argue the lack of such findings was merely due to the comparatively low affinity of fast skeletal myosin, so that movement associated with ADP release occurs too rapidly to be measured. Also, addition of ADP would not cause an ADP-bound state to become significantly populated and so movement would again not be visible in cryoelectron microscopy studies. However, studies by Yanagida’s group (in which chemical and mechanical states were observed simultaneously; Ref. 9)
imply that there might be little or no connection between ADP release and lever arm movement (and force production) in skeletal muscle. Structural studies of slow (tonic) smooth muscle, however, indicate that the working stroke may well occur in two phases (20), as observed with myosin I.

**Myosin Motors in Smooth Muscle Latch and Catch States**

Vertebrate smooth muscle myosin II probably does produce its working stroke in two phases (20), perhaps in a similar way to brush border myosin I. In cryoelectron microscopy studies, two states separated by ~23° axial rotation of the lever arm produced by ADP release were observed (20). According to Cremo and Geeves (4), opening of the ADP pocket to release ADP might depend on mechanical strain of the cross bridges. With a high load on the strained cross bridge, ADP release is slower or even prevented and the lifetime of the attached cross bridges is therefore extended. Clearly, a prolonged attached state (in which cross bridges are occupied by ADP) would provide a mechanism for economic tension maintenance in a holding function, e.g., in the so-called latch state of tonic vertebrate smooth muscle (11). This is important when we consider vascular smooth muscle that may maintain tension with little ATP consumption when withstanding a high blood pressure. In such a holding state, contractile force is supposedly supported by slowly cycling “latchbridges,” dwelling for a prolonged time in the intermediate attached state mentioned above as bound ADP is released and replaced by ATP only slowly. However, ADP release can be accelerated by phosphorylation of the regulatory myosin light chain, which increases the dissociation constant of the myosin-ADP complex and thereby shortens the intermediate attached state. In this way the latch state is terminated.

Recently Butler and colleagues (2) proposed that a long-lived intermediate attached state of myosin (i.e., an actomyosin-ADP state) may also account for the so-called catch phenomenon observed in certain molluscan smooth muscle such as the adductor muscles of bivalves, e.g., the scallop or the oyster, that may keep the shells closed for many hours and even days apparently without fatigue and barely any oxygen consumption. Another catch muscle, the anterior byssus retractor muscle of the edible mussel *Mytilus edulis*, develops an enormous tension (up to 15 kg/cm² cross section) when stimulated electrically or with acetylcholine but relaxes extremely slowly or even fails to completely relax after cessation of stimulation. Instead, one observes a remnant tension known as the catch, during which tension is maintained despite the fact that in this postcontractile state the cytosolic free calcium concentration reaches resting values (<0.1 μM). In catch there is little if any energy turnover and the muscle is held rigid, quasi in rigor, as if the cross bridges formed during the contraction preceding the catch were “frozen” in the attached state. Indeed, there is evidence (reported in Ref. 2) suggesting that, like the latch, the catch might well be due to the persistence of a locked actomyosin-ADP state or, for that matter, an intermediate attached state similar to that observed in myosin I. Thus actively contracting catch muscle may enter the catch state if its cycling cross bridges are trapped at low free calcium levels in a long-lived actomyosin-ADP state, stabilized perhaps by the high tension that pulls on the attached cross bridge. Furthermore, like the latch, the catch state too may be terminated by phosphorylation of a protein associated with the myosin-containing myofilaments. However, the myosin-bound protein concerned is twitchin (a kind of mini-titin) rather than the myosin light chains that are involved in the regulation of the latch state of vertebrate smooth muscle (2). Physiologically, this phosphorylation is brought about by protein kinase A, for instance in an oyster when its adductor muscles are stimulated by the neurotransmitter serotonin liberated from inhibitory nerves that eventually cause the shells to open.

**Perspectives: Functional and Structural Diversity of Myosin Motors**

In the preceding sections, we have given a short overview on how muscle myosin II isoforms are adapted to different functions such as quick movement or holding. Some muscle myosins are specialized for rapid shortening and are characterized by fast cycling and very short (some milliseconds) attached states and possibly perform the lever arm movement in one step. Because fast cycling requires a high ATPase activity, these motors are not economical for holding tension. Muscle myosins adapted for a holding function on the other hand are necessarily slow. In this case the attached state, probably an actomyosin-ADP state, is prolonged. Some of the slow unconventional myosins (e.g., brush border or liver myosin I) may even be capable of holding the cross bridge in a strain-dependent manner in an intermediate attached state for up to a second before completing the lever arm movement. Such diversities among myosins reflect functional specialization. The slow, single-headed liver cell myosin I, for instance, may be involved in linking the cytoskeleton to the plasma membrane, whereas fast, double-headed skeletal muscle myosin II interacts with hundreds of actin partners to produce fast filament sliding. At present, we know of at least 18 different classes of myosin in the plant and animal kingdom. Analysis of the human genome has shown that there are as many as 39 genes representing 10 myosin families. In the future, these motor proteins may be expressed and purified, and their mechanical properties could then be analyzed, specifically by using novel assays combining different nanotechnology techniques on the single-molecule level. As we have seen, these methods permit the mechanical properties of different myosins to be measured in an unequivocal way (16, 19). The importance of such studies is underlined by the discovery that mutations of some of these motor proteins can cause very severe symptoms such as deafness or seizures as well as cardiac failure due to inherited cardiomyopathy.

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*News Physiol Sci* • Vol. 17 • October 2002 • www.nips.org 217
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