THE MECHANISM OF MUSCLE CONTRACTION

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I. INTRODUCTION

It is now firmly established that the contraction of muscle and many of the movements of nonmuscle cells result from the interaction of two proteins, actin and myosin. These movements involve the relative sliding of two sets of filaments: the thick filaments, composed mainly of myosin; and the thin filaments, composed mainly of actin. It is generally accepted that the forces which cause filament motion are generated by cross-bridges that extend radially from the myosin filaments and interact cyclically with the actin filaments. The action of these cross-bridges has been extensively studied using a variety of physical and chemical techniques. However, in spite of an intense effort the actual molecular events that transduce chemical energy into mechanical energy remain largely unknown. This review will examine the experimental evidence that is most pertinent to our present understanding of this mechanism.

The structures of actin, myosin, and their complex are outlined in Section II providing a framework for discussions of the function of these proteins. The location of the contractile machine within these structures is then considered in Section III as defined by a variety of experiments involving the reconstitution of functional systems from purified and modified components. It is expected that force generation involves changes in protein structure, and the studies that have searched for such changes using purified proteins in solution are outlined in Section IV. A variety of techniques have been employed to measure cross-bridge orientation in muscle fibers. The results from such studies are discussed in Section V and an attempt is made to find a model of cross-bridge action that accounts for these findings. Finally, measurements of the kinetics of the contractile interaction in fibers and in solution are discussed in Section VI and used to define models of cross-bridge kinetics in the fiber.

There have been a number of recent books and review articles that are concerned with muscle structure and function. The topics covered include structural studies,[1-4] cross-bridge mechanics,[5-12] X-ray diffraction,[13-16] experimental techniques,[17] and kinetics.[18-22] Two good monographs on the field of contraction have recently been published.[23,24] The literature search extended to August, 1985.

II. STRUCTURES OF THE PROTEINS

A. Primary Structures of Myosin Subunits

Myosin is a large molecule composed of six polypeptide chains shown schematically

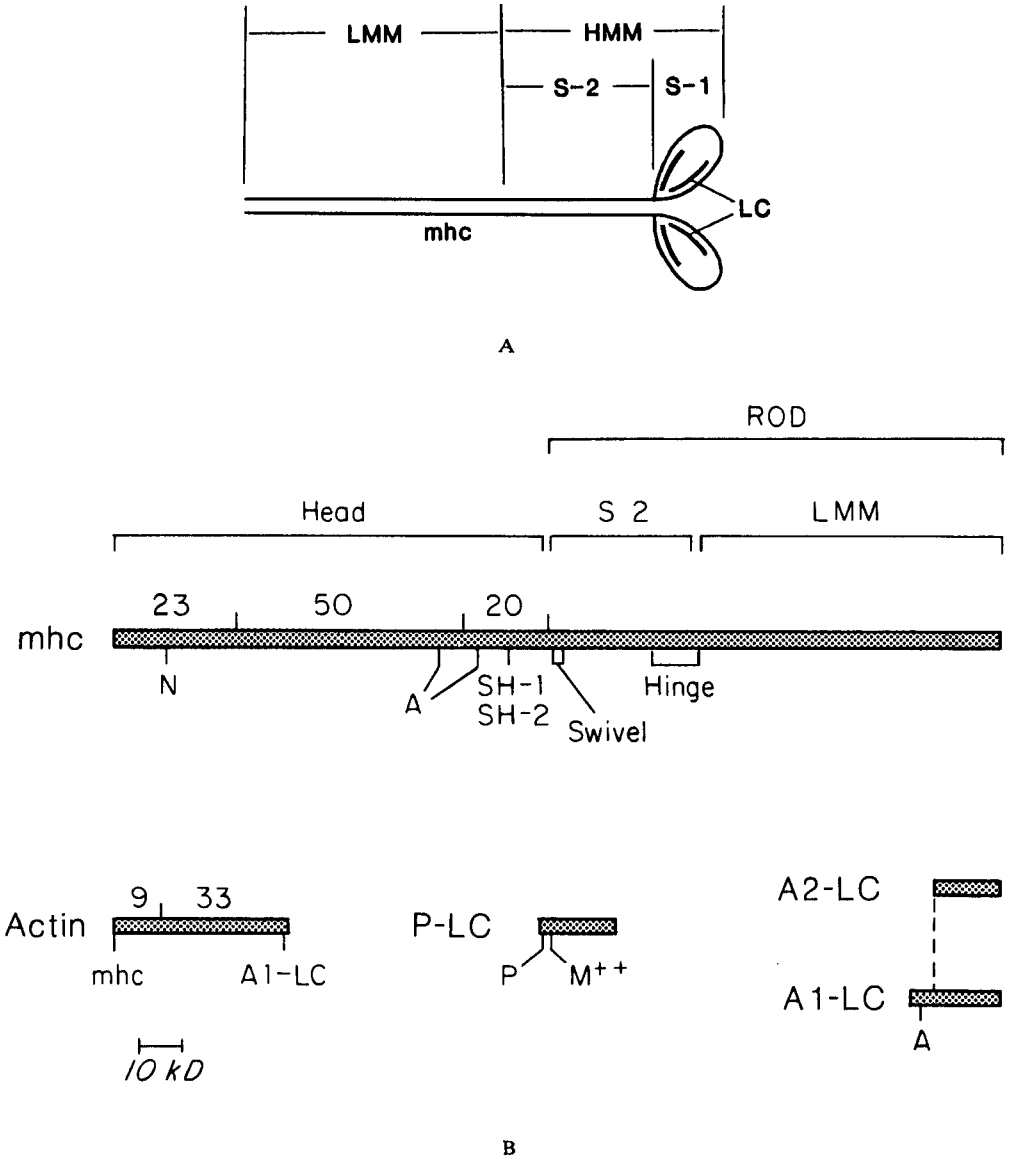

FIGURE 1. A schematic showing the polypeptides that compose myosin and actin, with known sites labeled; mhc is the heavy chain of myosin; A1-LC and A2-LC are the alkali light chains of myosin; and P-LC is the P-light chain of myosin. The chains are shown with their N-terminals on the left. Sites marked with N are known to be involved in the binding of nucleotide. Sites on the myosin chains that are marked with A can be cross-linked to actin in a rigor complex, and the corresponding sites on actin are identified by mhc and A1-LC. The sites for both divalent cation binding and phosphorylation are identified on the P-light chain. The inset above shows schematically how the two myosin heavy chains fold to form the intact molecule with two light chains (LC) on each head (S-1).

in Figure 1. It is highly asymmetric with a long tail which aggregates with tails from other myosin molecules to form the core of the thick filament, and two globular heads, which form the cross-bridges.[25,26] Two heavy chains, with a molecular weight of 220 kD, coil together in a helix of α-helicies to form the 150 nm tail region. This tail region, or myosin rod, is subdivided into two segments. A 86 nm section, light meromyosin (LMM), aggregates to form the thick filament, which is connected to the globular

heads via a soluble 60 nm section (S-2).[27] Each heavy chain also forms the bulk of one of the globular heads. In addition, each head contains two small polypeptide chains with molecular weights of approximately 20 kD. The two-headed myosin structure with attached tail appears to be highly conserved and is found in all cells from amoebae to humans. There is one exception: a single-headed "myosin" lacking a tail has been identified in acanthamoeba by its actin activated ATPase activity.[28] This soluble myosin may be involved in the movement of cellular organelles.

The complete amino acid sequence of two nematode *(C. elegans)* myosin heavy chains has been determined by sequencing the genes that encode them,[29,30] and rabbit skeletal muscle myosin has been sequenced by direct analysis of the polypeptide chain.[31,32] There is a high degree of homology among these three sequences. Analysis of these sequences has led to a number of predictions of the secondary and tertiary structures and to identification of the location of sites for binding nucleotides, probes, etc.

The sequence of that portion of the chain which forms the rod of myosin is highly repetitive and typical of an α-helical, coiled-coil protein.[30,33] There is a regular seven residue pattern in which hydrophobic amino acids are concentrated at alternate intervals of three and four residues along the chain. These residues form a hydrophobic stripe along one side of each helix and the two stripes bind together to form the interface between the two strands of the coiled coil. There is a distinct pattern to the distribution of charge along the rod so that strong attraction or repulsion will occur when two molecules are placed side by side. This attraction is enhanced when the displacement between two molecules is 14.5 nm, close to the distance between cross-bridges observed in the muscle. Thus, analysis of the amino acid sequence of the rod has provided at least a partial explanation of the forces that hold the core of the thick filament together.

There have been several proposals that the S-2 section of myosin may contain regions that are flexible or that can switch between helical and random coil structures, as discussed in Section IV. The sequence of the rod element strongly favors an α-helical secondary structure throughout its length, and there do not appear to be any extensive regions where the helical structure of the rod is particularly weak.[33] However, there are several locations in the rod where charged residues were found in the hydrophobic core between the two α-helices which would weaken the attraction between them.[34] One such section was found at the junction between LMM and S-2 where electron micrographs show that the myosin rod often forms kinks. A weaker bond between helices could introduce flexibility in this region that would allow the soluble S-2 fragment to swing out from the core of the thick filament.

Prediction of the secondary or tertiary structure in the globular head region is difficult; however, several interesting hypotheses arise from sequence comparisons among different myosins and between myosin and other proteins.[29,33] The homology among myosin heavy chains is much greater in the head region than in the tail region, there being an 82% homology between two myosin isotypes from the nematode and approximately 65% homology between one of these myosins and rabbit skeletal myosin. Limited proteolysis of the myosin head results in three discrete fragments with approximate molecular weights of 20, 50, and 23 kD (see Figure 1).[35] These sites of proteolysis were found to correspond to polar regions of 8 to 10 residues in length, which displayed a low degree of homology among the different sequences.[30,33] Such regions are often found to have rather mobile structures on the surfaces of proteins. In contrast, the sequences within the three proteolytic fragments are highly conserved.

Several sites of interest can be located in the sequence of the myosin heavy chain (see Figure 1). The N-terminal of the 23 kD fragment contains a region between residues 139 and 206 which is homologous with the nucleotide binding regions of adenylate

kinase and mitochondrial membrane ATP synthetase.[33,36] A glycine rich loop, residues 177 to 182, is also similar to sequences found in the nucleotide binding sites of other enzymes. The 23 kD fragment has been directly implicated in the binding of nucleotides by photochemical cross-linking of two nucleotide analogs, one of which binds to Trp-130.[37,38] There are several modified amino acids in the myosin sequence, and one of them, trimethyllysine, is also located close to this region at residue 128. Methylation of the nitrogen would enable a positive charge to be buried within the protein interior where it could conceivably interact with negative charges such as those on the phosphates of ATP.[32,33] In nematode myosin, the 50 kD fragment spans residues 212 to 645, and the 20 kD fragment extends from residues 655 to the "swivel". The swivel, which connects the head to the rod, appears to consist of an unconserved sequence characteristic of a random coil spanning residues 808 to 824.[30,33] This region most probably provides the flexibility between head and rod that is necessary for the function of myosin. The 20 kD fragment contains two reactive sulfhydryls that have been extensively used as sites for fluorescent and paramagnetic probes. In rabbit myosin the more reactive residue, known as SH_1, is found a position 707 and the less reactive SH_2 at 697. The 10 residues which link these two groups appear to be flexible, a property that may explain the observation that nucleotide binding greatly alters the distance between SH_1 and SH_2 (discussed in Section IV).

The 20 kD tryptic fragment forms a structural entity that binds both of the light chains. A bacterial protease clips scallop myosin at the junction between the 20 kD and 50 kD fragments in such a way that the 20 kD fragment can be isolated while still attached to the rod.[39] Electron microscopy of this fragment showed a rod which terminated in two nubs each about 10 nm in length. The nubs were comprised of the 20 kD fragments and the two pairs of light chains. In another experiment, the 20 kD fragment from skeletal myosin was purified by column chromotography and renatured into a structure that retained the ability to bind to actin with high affinity.[40] The above experiments show that the 20 kD fragment forms a structural unit relatively autonomous from the 50 kD and 23 kD fragments. As discussed below, this unit extends from the head-rod junction to the sites that interact with actin. There is no evidence that either the 50 kD or 23 kD fragments form structural domains.

One question, crucial to understanding the function of myosin, has been concerned with its two-headed structure. A large body of experimental evidence has suggested that the two heads of skeletal myosin have different properties.[41] Even within a single muscle cell there are at least two isozymes of the myosin heavy chain leading to the possibility that there may be two functionally different heads on each myosin molecule. To determine whether the two heavy chains of a single myosin molecule came from different genes, myosin molecules were isolated from muscles of *C. elegans* in which one of the myosin heavy chain isozymes carried a 20kD deletion at the N-terminal end.[42] The majority of the myosin molecules from these muscles were homodimers, showing that the two heads on each myosin can be formed from heavy chains coded by the same gene. Any differences between the two heads are then relegated to post-translational modifications, to the association of different isozymes of the light chains, or to an induced difference when the two heavy chains associate. This evidence, along with evidence from studies of function discussed in Section III.B, strongly suggests that the two heads function independently in the generation of force. Although most muscle cells appear to produce at least two different forms of myosin heavy chains, the function of this diversity remains obscure. One clue to this function may be contained in the observation that in nematode thick filaments one myosin isozyme was confined to a central 1.8 μm zone while the other isozyme formed the rest of the filament.[43]

Each myosin head contains one each of two classes of light chains.[44,45] Although the sequences of these classes are distinct from each other, they are both homologous with those of a wider class of calcium-binding proteins that also includes troponin, parvalbumin, and calmodulin.[46] One class of light chain, mol wt 19 kD, can be phosphorylated and has a single site for binding divalent cations. This class has been referred to as the DTNB light chain and more recently as the phosphorylatable or P-light chain. This light chain is clearly involved in the regulation of contraction in a variety of muscles. In some molluscan muscles, regulation is achieved by the binding of calcium directly to the P-light chain,[45] although calcium binding does not appear to play a role in the regulation of other muscles.[47] In smooth muscle and some nonmuscle cells, regulation is exerted via phosphorylation of a specific serine residue near the N-terminal end of this chain,[45,48] and in skeletal and cardiac muscle phosphorylation modulates the contraction.[48] The second class of light chain was previously misnamed the essential light chain, and is here termed the alkali or A-light chain. It was first thought that this light chain was essential for myosin ATPase activity, but it now appears that myosin heavy chain when devoid of both light chains still retains an actin activated ATPase activity similar to that of intact myosin.[49,50] In fast skeletal muscles there are two distinct isoforms of the alkali light chains, and it has been found that these can bind to myosin to form both hetero- and homodimers. The two isoforms are produced from the same gene by differential splicing of the mRNA to add additional sequences to their N-terminal ends.[51] The isoforms are both present within single muscle fibers, and appeared to be evenly distributed along the thick filament.[52] Both classes of light chains can regulate the actomyosin interaction, and their function is discussed more fully in Section III.C.

B. The Shape of the Myosin Head

Electron micrographs of rotary shadowed molecules of myosin showed a head that is 17 to 20 nm in length, measured from the tip of the head to the Y junction, where the two heads join to form the rod.[53] The head was roughly pear shaped with its greatest width, approximately 7 nm, near its tip. The head tapers to about 3.5 nm at the junction between S-1 and S-2. A similar head shape with slightly larger dimensions was observed in isolated negatively stained myosin molecules.[54] When negatively stained intact myosin filaments were viewed on hydrophilic grids, many myosin heads could be seen splayed out on the grid surface, as shown in Figure 2.[55] Most of the heads were slightly curved and they had dimensions that roughly agree with the earlier work which viewed single rotary shadowed myosin molecules. However, the dimensions obtained in these above studies were somewhat uncertain because the myosin heads appeared to have been flattened onto the surface during preparation for viewing.

The myosin heavy chain can be cleaved in the region of the swivel by proteolytic enzymes to produce isolated heads, known as myosin subfragment-1 (S-1). The shape of S-1 was first deduced from electron micrographs of actoS-1 complexes.[56-58] The results depended upon the presence of the P-light chain (see Figure 5). Reconstructions using S-1 molecules that lacked the P-light chain showed an S-1 with a contour length of 12 to 15 nm, considerably shorter than seen for the heads of myosin.[57,58] In the presence of the P-light chains S-1 appeared curved, with a contour length of about 17 nm.[57] Both the shape and the dimensions of this S-1 agree approximately with those found for the heads of intact myosin; however, the resolution of either structure, approximately 3 nm, does not allow a detailed comparison. These observations suggest that in the absence of the light chain the portion of the myosin head distal to the actin site is thinner and possibly more disordered.

Recently S-1 has been crystallized in a form suitable for high resolution structure

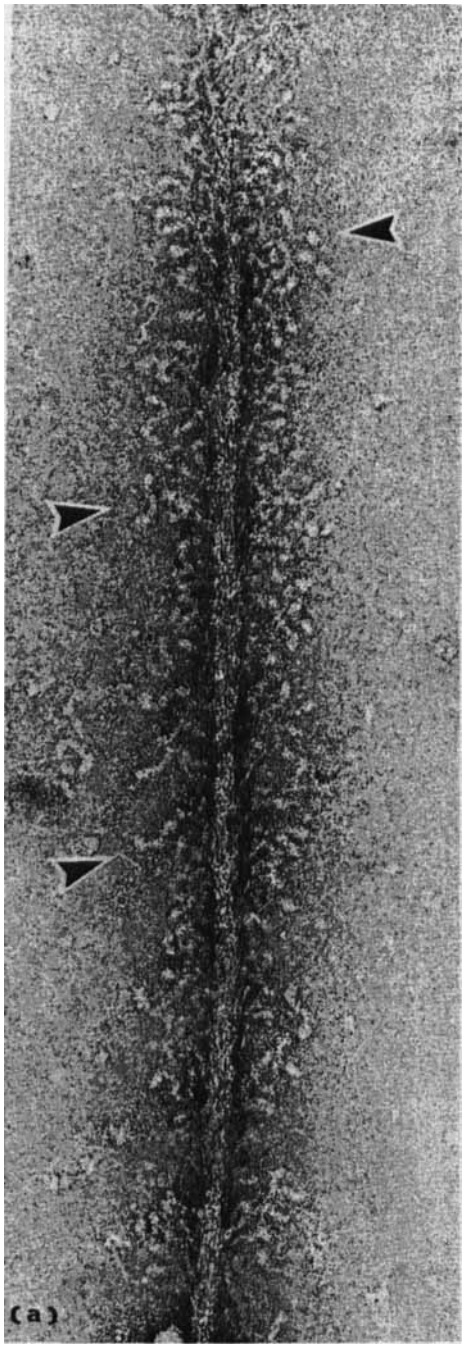

FIGURE 2. Portion of negatively stained thick filament. Isolated filaments from rabbit psoas muscle were applied to hydrophilic carbon films and stained with uranyl acetate. A fringe of myosin heads surrounds the filament backbone (arrows point out prominent examples). (Magnification x 215,000.) (From Knight, P. and Trinick, J., *J. Mol. Biol.*, 177, 461, 1984. With permission.)

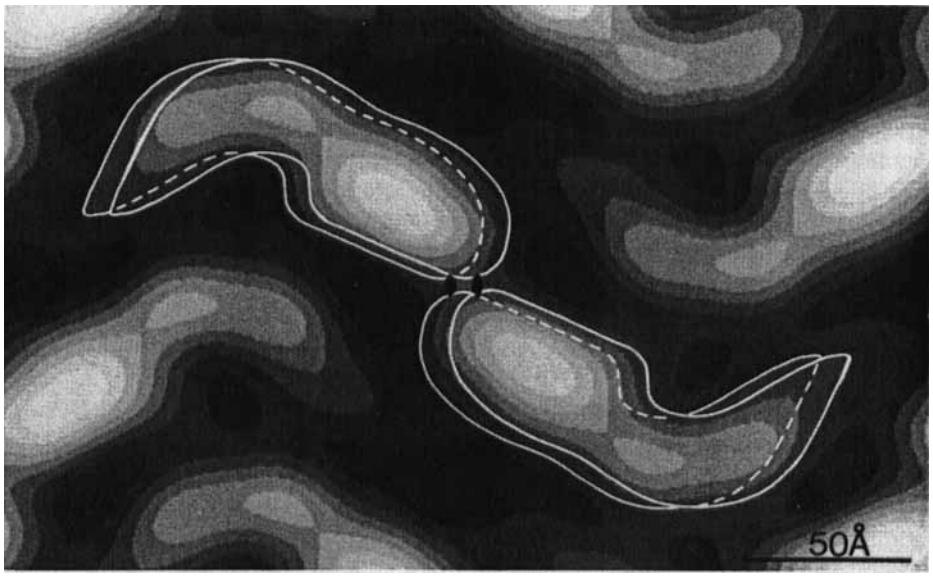

FIGURE 3. A filtered image of an electron micrograph of crystals of myosin subfragment-1. Thin sections were cut parallel to the {100} plane from tannic acid-embedded crystals. The stain-excluding light regions represent the protein. Two superimposed dimers are outlined by solid and dashed lines. The contour levels reflect a cutoff that underestimates the size of the molecule. In the crystal, adjacent molecules must make contact and the myosin head is very probably larger than shown here. The crystallized subfragment-1 contained both light chains. (From Winkelmann, D. A., McKeel, H., and Rayment, I., *J. Mol. Biol.*, 181, 487, 1985. With permission.)

determination,[59] and the shape of S-1 has been resolved using image analysis of electron micrographs of the three crystal faces (see Figure 3).[60] The molecule has a prominent mass at one end that is about 6 nm in length and 4 nm in width. The thickness of the molecule perpendicular to the plane shown in Figure 3 is no greater than 5.4 nm. A curved region of lower density extends from one end of this mass, and this thin curved "neck" is separated from the prominent mass by a region of still lower density. The contour length of the entire molecule is about 16 nm, which is probably an underestimate because the tip of the thin neck region is not well defined. A comparison of the image shown in Figure 3 with those of intact myosin heads and acto S-1 complexes leads to the conclusion that the thin neck region terminates in the connection to the rod while the more massive region binds to actin. The S-1 shown in Figure 3 contains both light chains and its shape and dimensions agree roughly with those obtained from reconstructions of an acto S-1 complex shown on the left in Figure 5. The crystallization of S-1 is an exciting breakthrough that is the first step to obtaining the high resolution structure of this protein. Such structures for both S-1 and actin will be an essential component of any detailed understanding of the molecular mechanism of contraction.

The shape of S-1 in solution has been determined by small angle X-ray scattering.[61,62] The data from these experiments can be analyzed to give two parameters that are independent of the assumed shape of the S-1: the radius of gyration, 3.2 ± 0.1 nm, and the maximum chord, around 12 nm. Both of these parameters were smaller than those obtained from electron micrographs of intact myosin. More information on the shape of S-1 can be obtained by comparing the scattering data with simulated data from various models of the S-1 shape. Good agreement was obtained with the shape of S-1 deduced from acto-S-1 complexes lacking the P-light chain.[61] However, the shape of

the scattering curve obtained for S-1 deviated significantly from the scattering expected from the longer myosin head visualized by rotary shadowing.[61,62] The discrepancy between the shapes of S-1 determined by different techniques could have several explanations. One possibility, that S-1 changes shape when it is cleaved from myosin, has been eliminated by comparing the X-ray scattering from single-headed HMM and from the same protein digested to S-1 and S-2.[62] No change in scattering was observed following the digestion, indicating that cleavage of the junction between S-1 and S-2 did not cause a change in the shape of S-1. A second possibility is that the narrow neck region of S-1 will make a reduced contribution to the overall scattering pattern and thus may lead to an underestimate of the total length of the head.[62] This possibility is supported by the shape of S-1 determined from crystals which show a rather narrow neck region whose distal portion is not well resolved in the crystals and could be missed in the diffraction data.

Several lines of evidence now indicate that both classes of light chains have one end located on the myosin head close to the region where the head connects to the rod. Scallop myosin has an easily extractable P-light chain, and the shape of the scallop acto S-1 complex, as seen in the electron microscope, was altered upon removal of the P-light chain.[63,57] Note, although phosphorylation of this light chain in scallop has not been demonstrated, it is homologous with the phosphorylatable light chains of other myosins and will be designated as the P-light chain. Reconstructions of these complexes showed that inclusion of the P-light chain resulted in an increased mass in the neck region of S-1.[57] When individual molecules of scallop myosin were resolved in electron micrographs, the presence of the P-light chain was again seen to enhance the density of the region where the head and rod were connected.[64] Antibodies specific for both the P-light chain and the A-light chain were prepared and their complexes with myosin observed in the electron microscope.[64] Antibodies specific for both light chains were observed to bind to a region that extended from the "Y", formed by the junction of the two heads and the rod, to about one half of the length of the myosin head. Very few fragments bound to the rod or to the 8 nm of the myosin head most distal to the rod.

In scallop myosin there appears to be considerable contact between the P-light chain and the A-light chain. The presence of the P-light chain protected the thiol groups of the A-light chain.[65] Cross-linking of the two light chains *in situ*, with photo-activated bifunctional reagents showed that they are in close proximity for at least half of their lengths.[66] The N-terminal regions of the P-light chains on adjacent heads of scallop myosin can be cross-linked to each other, and electron micrographs showed that the cross-linked regions of the light chains are close to the head-rod junction.[67] Antibodies that reacted with the divalent metal ion site near the N-terminal end on the P-light chain were found to bind to the head-rod junction of chicken myosin.[68]

Thus the two light chains along with the 20 kD heavy chain fragment appear to form the portion of the myosin head that is proximal to its junction with the rod. The length of this domain is such that it could comprise most, if not all, of the thin neck region resolved in electron micrographs of S-1 crystals.[39] However, both the 20 kD fragment and the A-light chain also bind to actin, and since actin is thought to bind near the other end of the myosin head, these chains must extend for a considerable distance along the myosin head.

C. Structure of Actin and the Actin Filament

Actin is a globular protein with a single polypeptide chain, which in skeletal muscle is composed of 375 amino acids.[69] At physiological salt concentration, actin polymerizes into a double-stranded helical array that forms the core of the thin filaments, shown in Figure 4. Each actin monomer interacts with three to four other monomers

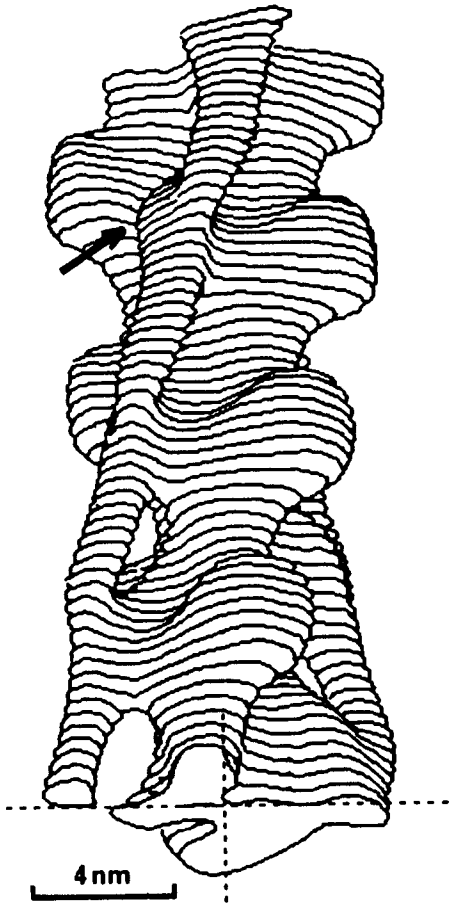

FIGURE 4. A three-dimensional reconstruction of an actin·tropomyosin complex obtained from data derived from electron micrographs of paracrystals. The arrow marks the smaller domain of the actin, to which tropomyosin is attached. The dotted cross marks the position of the filament axis. The structure shows an obvious polarity which in this picture is oriented such that the Z line would be below. (From O'Brien, E. J., Couch, J., Johnson, G. R. P., and Morris, E. P., *Actin Structure and Function in Muscle and Non-Muscle Cells*, dos Remedios, C. G. and Barden, J. A., Eds., Academic Press, Sydney, 1983, 3. With permission.)

in the polymer. In addition to these interactions, actin forms bonds with the two regulatory proteins, troponin and tropomyosin, and with numerous other proteins which mainly regulate polymerization in nonmuscle cells.[70] The most important active sites in actin, of course, are concerned with its force-generating interaction with myosin.

The sequences of actins from a variety of muscle and nonmuscle cells have been determined and show a remarkable degree of homology. Typically only a few amino acid substitutions were found, and these were conservative and largely confined to a small region near the N-terminal end of the chain.[71] The high degree of conservation in the actin primary structure reflects the fact that it evolved to its present form very early in the development of eukaryotic cells. The large numbers of binding sites on this relatively small molecule may constrain further structural alterations.

Limited proteolysis of actin has suggested that there are two structural domains. A number of enzymes clip the actin monomer into a 33 kD C-terminal fragment and a small easily degraded 9 kD N-terminal fragment.[72,73] Each actin monomer has a tightly bound nucleotide and cation. The nucleotide binds to the 33 kD fragment [73] while the cation site is probably located within the smaller fragment. Neither fragment retained the ability to polymerize or interact with myosin.[72,73]

Actin has been crystallized only when complexed with small molecules that prevent polymerization. Crystals of two such complexes, with profilin or DNase I, have now been formed and examined by X-ray diffraction.[74,75] The crystal of the actin DNase complex has been determined to 5 Angstrom resolution.[74] The difficulty of separating the DNase molecule from the actin has been removed by crystallizing DNase alone and solving its structure. The 6 Angstrom structure of actin shows that the molecule is asymmetric with dimensions of approximately 7 × 4 × 3 nm. It is composed of two domains, one larger than the other. The domains are separated by a deep cleft that is thought to contain a tightly bound nucleotide. The correspondence, if any, between these two structural domains and the regions revealed by proteolysis is unclear.

The structure of the F-actin polymer has been elucidated by analysis of electron micrographs and by low angle X-ray diffraction from the thin filaments of muscle fibers. The helical array of actin monomers can be described by a single left-handed helix with a pitch of 5.9 nm. Alternatively the structure can be described as two right-handed helical strands intertwined with crossovers every 36 to 38.5 nm. Recent reconstructions of actin polymers, shown in Figure 4, have resolved the actin monomer into a major domain and a minor domain,[76] similar to those determined from X-ray crystallography. The long axis of the actin monomer appeared to be oriented roughly perpendicular to the filament axis. The width of the filament was approximately 9.5 nm. The connection between actin monomers along the left-handed helix appeared strong in the reconstruction while more tenuous connections linked actin monomers along the right-handed long pitch helices. A similar model of the actin filament has also been derived from analysis of the X-ray diffraction pattern of molluscan smooth muscle.[77] The surface of actin filaments has been viewed in the electron microscope using a rapid-freeze-etch technique revealing a 8 to 10 nm filament with a prominent left-handed helix having a 5.9 nm pitch.[78] This view of the actin structure is similar to the surface of the filaments seen in reconstructions of negatively stained filaments.[79,80]

Attempts are now underway to fit the actin structure, determined by crystal diffraction, into the polymer structures visualized in the electron microscope. As discussed above, the long axis of the actin monomer in reconstructed actin filaments appears to be roughly perpendicular to the filament axis. The structure of the actin monomer has been determined using actin sheets formed in the presence of gadolinium,[81,82] and an orientation more parallel to the actin filament was favored from this structure.[82] The parallel orientation would result in a narrower filament than would the perpendicular orientations. Recently the maximum width of the filament was found to be 10 nm using X-ray diffraction, thus favoring the perpendicular orientation.[83]

Analysis of electron micrographs of negatively stained actin filaments showed that there were significant differences between the observed positions of actin monomers and the positions expected for a regular helical array.[84,85] Although a high degree of axial order existed, the twist of the long pitch actin helices could vary. The observed helical arrays can be explained if the angular displacement between monomers in the 5.9 nm left-handed helix is 167° with a random variability of ±10°. This variability did not appear to be caused by sample preparation for electron microscopy, although the exact contribution of preparative procedures has not been evaluated. The Brownian rotations of actin monomers within the polymer have been measured using both paramagnetic and phosphorescent probes attached to Cys_{374}.[86,87] Both probes detected mo-

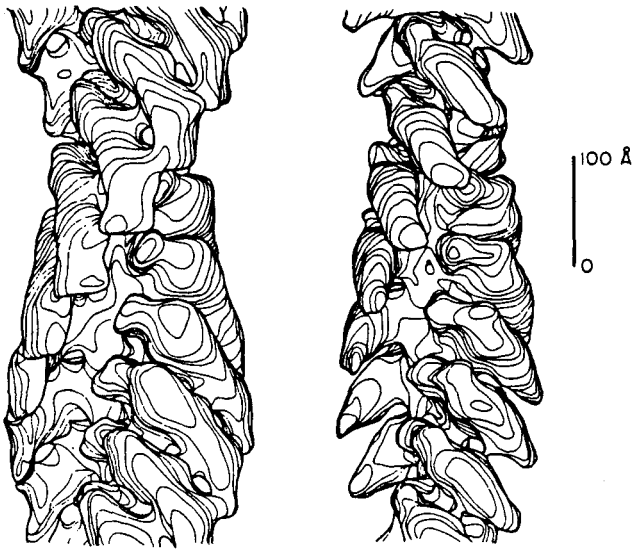

FIGURE 5. Two three-dimensional reconstructions made from electron micrographs of acto S-1 complexes. The complex, shown on the left, was obtained using S-1 from scallop muscle that contained both light chains, while that shown on the right contained S-1 that lacked the P-light chain. The view is perpendicular to the axis of the helix. In the absence of the light chain the S-1s are shorter by about 4 nm and lack the portion most distal from actin. (From Vibert, P. and Craig, R., *J. Mol. Biol.*, 157, 299, 1982. With permission.)

tion in the microsecond range which was compatible with the torsional flexibility seen in electron micrographs.

In summary, the actin filament is composed of a two-lobbed elongated monomer with its long axis roughly perpendicular to the filament axis, thus forming a prominent 5.9 nm left-handed helix. There appears to be considerable flexibility which allows azimuthal rotations of the positions of the monomers about the filament axis. These rotations allow the pitch of the long pitch helix to vary without permitting stretch. The angular flexibility of actin may play a role in contraction by making it easier for myosin and actin to interact within the geometrical constraints imposed by the filament lattice of the muscle fiber. This possibility is also suggested by electron micrographs of rigor fibers which show that the actin filament helix is distorted considerably by its interaction with the myosin cross-bridges (discussed in Section V.A).

D. The Structure of the Actomyosin Complex

In the absence of nucleotides, actin and myosin form a very tight complex, which is thought to represent the state that occurs near the end of the powerstroke in the cycle of cross-bridge states that generate force. This complex is highly ordered and has been studied extensively leading to a structure with about 3 nm resolution, see Figure 5. The original reconstructions of acto-S-1 polymers led to the conclusion that S-1 was elongated and curved.[56] It bound to actin so that it was both tilted and slewed with respect to the filament axis. New techniques, including low dose electron microscopy, and the use of X-ray diffraction patterns obtained from actin decorated with S-1 in muscle fibers, have led to an increased resolution of the complex.[58] These pictures showed a comma-shaped S-1 with a broad head which interacts with actin, possibly near the

groove between the two strands of the actin helix. The myosin head appeared to make contact with two actin monomers in the filament, which were adjacent to each other in the left-handed helix of actin. S-1 has a region adjacent to actin that is approximately perpendicular to the filament axis, however, the major portion of the S-1 is tilted at about 45° to the filament axis and slewed around the filaments, as shown in Figure 5.[57] Both actin and S-1 are composed of a number of different domains.[81,82]

These pictures of the myosin head obtained from fully decorated filaments can be compared to views of single S-1s or HMMs bound to actin filaments and visualized by negative stain.[63] The single S-1s have a principle axis that is at an angle of about 45° to the filament axis. The heads of HMM appear to attach to adjacent actin monomers on the same long pitch helical strand of the filament. If one end of each head binds in the same stereospecific manner to different actins then considerable distortion of the heads must be introduced in order for their other ends to join together at the head-rod junction. This distortion appears to involve an elongation and bending of the leading head.[63]

The use of bifunctional reagents to form cross-links between adjacent polypeptides has provided information on the location of the various regions of myosin and actin that participate in their interface in the complex. The use of a chemical cross-linking agent, EDC, which is a water soluble carbodiimide that catalyzes the formation of a covalent bond between adjacent amino and carboxyl groups, ensures that the two linked chains formed close contacts.[90] After cross-linking the actomyosin complex, myosin was cleaved into its three tryptic fragments and the locations of cross-links were determined. It was found that the N-terminal residues at positions 1 to 11 of the actin sequence were cross-linked to two regions that flank the junction between the 20 and 50 kD fragments.[90-92] Additional evidence that actin interacts with this region comes from the observation that the binding of actin protected the 20 to 50 kD junction from proteolysis.[93] Conversely, cleavage of this junction greatly lowered the affinity of actin for S-1 in the presence of ATP.[94] Several recent investigations have all reached the conclusion that the stoichiometry between cross-linked actin and myosin is 1:1.[91,95,96] In addition, the A-light chain of myosin can be linked to the C-terminal region of actin.[97] The foregoing studies show that the 20 kD fragment of S-1 must span a considerable distance within the myosin head. The C-terminal end of this fragment forms the junction with the rod while its N-terminal end interacts with actin.

In summary, the primary structures of both actin and myosin have been determined and a number of important sites identified. The structures of the myosin head, the actin filament and their complex are now known to a resolution of approximately 3 nm, which is insufficient to determine how these proteins function. However, along with electron micrographs of muscle fibers, the tilted and slewed orientation of S-1 bound to actin suggests that a change in orientation may have occurred during the generation of force. Structural data acquired from analysis of protein crystals will be required to understand the function of these proteins. As discussed above such data may soon be available from crystals of both actin and S-1.

III. WHERE ARE THE ELEMENTS RESPONSIBLE FOR FORCE PRODUCTION LOCATED?

A. Studies of the Filament Array of Muscle

Cross-bridges connecting thick and thin filaments were first observed in 1953,[98] and shortly afterward two experimental results suggested that they were the elements responsible for force generation. The first of these was the observation that muscle shortening was the result of the relative sliding of two sets of constant length filaments.[99,100] The second was the observation that the force generated by the filaments was propor-

tional to the overlap between the actin filaments and the myosin cross-bridges.[101] Although a vast body of evidence now supports the conclusion that force is generated by the interaction of the cross-bridges with the thin filaments, both of the above observations have recently been questioned in light of new experimental evidence (reviewed in Reference 5).

The original electron micrographs obtained from skeletal muscle at different sarcomere lengths led to the conclusion that both myosin filaments and actin filaments maintained a constant length;[99,100] however, some more recent work has questioned whether this is universally true. In particular, considerable A-band shortening was found to accompany sarcomere shortening in Limulus muscle.[102] In contrast, Levine et al.[103] recently found that the axial cross-bridge periodicity of isolated limulus filaments was a constant independent of the length of the filaments. No plausible mechanism suggests itself to explain thick filament shortening that would produce force while maintaining a constant subunit repeat, and the observed changes in A-band width may be due to misalignment of the thick filaments.[104] It thus appears that force is not generated by thick filament shortening.

The observation that the tension of a muscle fiber depended linearly on the overlap between the thin filaments and the cross-bridges has also been recently questioned. The tension measured at longer sarcomere lengths was greater than expected, as reviewed in Reference 5. After considerable debate it appears that most of this discrepancy disappears when the sarcomere length is maintained constant.[105,106] Although, in some instances an exact correlation is still not obtained, these data are consistent with force generation via myosin cross-bridges.

B. Studies of Reconstituted Contractile and Motile Systems

Further definition of the location of the force-producing elements can be achieved by a study of the interaction of myosin or its subfragments with actin. The ATPase activity of actoS-1 is very rapid and is approximately equal to the maximum activity of isotonically contracting muscle.[18,23] This observation shows that the mechanism for rapid ATP hydrolysis is contained within the complex of a single myosin head and actin. However, in such an assay no force is measured, and thus it is possible that a significant portion of the force-generating mechanism has been lost. To ensure that one is dealing with an intact force generator one must turn to experiments in which the generation of force is measured.

Early studies showed that force generation did not require the highly organized array of the muscle fiber.[107] Solutions of actomyosin could be aggregated into long thread-like structures that could generate force. Subsequent work showed that the force generated by such threads could be reproducibly and quantitatively measured.[108] Force could be generated by threads formed from highly purified actin and myosin, eliminating the possible involvement of other minor protein components that were present in the original studies. The force generated by the threads was small; however, the protein concentration in the threads was low and the force generated per myosin head was high. Such threads were used to decide whether both heads of myosin are necessary for force generation.[109] Threads formed from single-headed myosin generated the same tension per head as did threads of two-headed myosin. This observation led to the conclusion that the two heads operate independently in the generation of tension, a conclusion that is compatible with the structural studies of myosin which indicate that the two heads of myosin are probably identical. Although the threads appear to be a good system for assessing the tension-generating ability of purified proteins, several results show that they are not a reasonable system for measuring the ability to produce motion in muscle. The velocities of isotonic contractions were very low and they were

faster at higher protein concentrations.[109] In addition, myosin extracted from fast muscles and from slow muscles produced the same velocities of contraction.[110] The rate of contraction is probably inhibited because relatively few myosin heads are operating against a rather high internal viscosity in the disordered filament array of the thread. Fortunately, a motile system capable of measuring rapid velocities has recently been developed as described below.

A rapid and quantitative assessment of the ability of myosin to generate motion can be gained by monitoring the movement of myosin coated beads along bundles of actin filaments.[111,112] The interior walls of the cells of a giant algae, Nitella, are coated with bundles of actin filaments which all possess a well-defined polarity. Rabbit muscle myosin and its subfragments were attached to small fluorescent beads (diameter 0.7 μm). When these beads settle on to the actin substratum, individual beads coated with rabbit skeletal myosin can be seen to move along the actin with velocities ranging from 3 to 5 μm/sec, close to the maximum rate of filament sliding measured in rabbit psoas fibers. Motility required ATP, and both myosin and HMM produced similar rates of movements. These results suggest that a myosin filament is not required for producing motion. Because the HMM used in these studies was made by a method which results in a short S-2 region, these results also suggest that neither LMM nor the proteolytically sensitive region between S-2 and LMM are required for contraction. More recently it has been found that preparations of HMM highly purified by sedimentation do not generate motion in the beads, suggesting that some form of protein aggregate may be required for this interaction.[112] Addition of small amounts of myosin to these preparations did not reconstitute the motility of the beads showing that the proposed aggregate was not a myosin filament. Although these complications raise some doubts, two recent results strengthen the conclusion that a filament is not required for mobility. A soluble myosin from Acanthamoeba, which lacks a tail region, produced bead motility,[113] and antibodies that bound to soluble myosin but not to filaments inhibited mobility.[114]

Under some conditions motion or force appears to be generated by the interaction of actin filaments and soluble myosin subfragments. Myosin can be extracted from myofibrils or glycerinated fibers resulting in preparations that do not contract. Addition of either S-1 or HMM in the presence of ATP could contract these myofibrils and generate a low tension in the fibers.[115] A motor driven by actin and myosin subfragments has been recently reconstituted.[116] Actin can be polymerized using polylysine coated surfaces such that the actin polymer has a well-defined polarity with the arrowhead of decorated actin pointing towards the polylysine surface.[117] When sheets of actin filaments were oriented on polylysine, the fluid adjacent to the surface flowed in the presence of ATP and myosin subfragments.[118] A more spectacular machine was then constructed in which actin filaments were attached to the trailing face of each of four paddles connected to a rotor.[116] In the presence of both ATP and myosin subfragments the rotor revolved very slowly in the same direction as the arrowheads of the attached actin, i.e., the actin pushed on the face to which it was attached.

If soluble myosin subfragments can in some way deliver momentum to an actin filament, one can draw several conclusions concerning the mechanism of contraction. Within the framework of current models the most reasonable explanation is that a myosin head, or at least a large portion of the head, rotates while attached to actin. The rotation could deliver momentum to the surrounding fluid in the other direction. These results thus argue for a rotating cross-bridge, a concept that is still controversial, as will be discussed later. In addition, the results argue that the mechanism for producing this thrust is contained completely within one myosin head and the actin filament.

However, each of the above experiments leaves unanswered questions. Myosin can never be completely extracted from fibers, and tension generation by residual myosin

is difficult to eliminate. The tension generated in ghost fibers by subfragments was weak and the possibility of myosin subfragments binding to residual thick filaments can not be excluded. The hydrodynamics of these systems are complex, and as yet have not been analyzed. For instance, in the paddle machine an actin filament whose length is of the order of one micron is attached to a plate that is 1 cm wide. To obtain a feeling for the geometry involved, the dimensions are equivalent to those of stalks of corn 1.5 m high standing in a field that is 16 km on each side. An actin filament near the center of such a plate is pushing against a surface which is effectively infinitely wide, and it would be difficult to deliver a net momentum to the water in such a geometry. Thus the effective region for producing force must lie only along the edges of the plate. Further questions are raised because the actomyosin motor rotated with a rather surprising speed. The outer edge of the paddles moved at approximately 40 μm/sec, 10 times the maximum velocity of filament sliding measured in a fiber.[116] It is surprising that the interaction of HMM with a limited fringe of actin at the edge of the plate would transfer sufficient momentum to the fluid to move a large plate through a viscous medium at the observed velocities. Further work designed to discover the mechanism of force production by soluble myosin subfragments would help in interpreting the current results. An alternative explanation of force production, which would not involve cross-bridge rotation, has been formulated by Oosawa et al.,[119] discussed below. A nonrotating S-1 generates transverse oscillations in actin which may in turn impart momentum to the fluid.

Recently, the motion of single actin filaments has been viewed using dark field microscopy, and the amplitude and frequency of Brownian vibrational modes were directly measured.[119] When HMM and ATP were added the bending motion of long actin filaments became faster and larger in amplitude. These motions require energetic input from ATP hydrolysis. If the stiffness of the filaments had changed, e.g., became more flexible, the amplitude of vibrational motion would increase while the frequency of the vibrations would decrease. This was not observed and the vibrational modes of the filament now contain more energy, thus an active transduction of chemical to mechanical energy has occurred. An important observation is that no axial motion of the filaments was generated. Thus the interaction with these soluble myosin fragments can transduce chemical energy into increased vibrational energy but not translational kinetic energy. The lack of translational motion of the actin filaments raises further questions about the mechanism of force generation in the actomyosin motors discussed above.

C. Active Elements within Myosin and Actin

Several experiments have suggested that the machinery for producing force is contained completely within the heavy chain of myosin and that the light chains play a role in regulating this interaction. Removal of either the P-light chain or both light chains did not abolish the actin activated ATPase activity of S-1, [49,50] and removal of the P-light chain did not affect the ability of myosin to generate tension in actomyosin threads.[120] A regulatory role for the light chains in vertebrate striated muscle has been suggested by a variety of results including: (1) removal of the P-light chain altered the calcium sensitivity of regulated actomyosin[121] and decreased the velocity of glycerinated fibers;[122] (2) the presence of an intact P-light chain increased the ability of troponin and tropomyosin to regulate the binding of HMM;[123] and (3) different isozymes of the A-light chain produce different actoS-1 ATPase activities at low ionic strength although these differences disappear at physiological ionic strength.[124,125] The major difference between these two isozymes is an extra 41 residues on the N-terminal end of the Al-light chain, and two results indicate that this segment interacts with actin. The

Al- but not the A2-light chain can be cross-linked to actin,[91,92] and the mobility of this segment is markedly reduced by the binding of actin, discussed in Section IV.B.

The inhibition of function by antibodies directed to specific myosin sequences has also been used to locate essential elements.[114,119] As expected, some antibodies to the head region inhibited actomyosin ATPase and the contraction of actomyosin gels. However, myosin function was also inhibited by a group of antibodies that bound to S-2 adjacent to the head region. Unexpectedly, an antibody that bound to the tail region 120 nm from the heads inhibited the motility of the myosin coated beads.[114] The mechanism of inhibition by these antibodies is not known and could arise because the bound antibody indirectly blocks some required movements of another region. However, these results do suggest that a region of S-2 adjacent to S-1 forms a portion of the force-generating machinery. Further work will be required to distinguish the mechanism of antibody induced inhibition.

Actin has usually been assigned a more passive role than myosin in force generation. There is, however, no definitive evidence to support such an assignment. On the other hand there is little evidence that defines the function of actin or localizes regions of actin that are involved in the generation of force. Monomeric actin is not a good activator of myosin ATPase; however, it is not known whether force generation requires more than one monomer in a filament.[127] One possibility for an active element within actin is the nucleotide that is tightly bound to each monomer. F-actin that has had a large portion of its bound nucleotide removed was capable of generating full tension in actomyosin threads indicating that the nucleotide is not required for the actomyosin interaction.[128]

D. Summary

There is good evidence that force can be generated by the interaction of a single myosin molecule with one or more actin monomers in an actin filament. The heads operate independently in this interaction, and the force generating mechanism is most probably formed by the heavy chain. The generation of motion by actin filaments and soluble S-1 in a variety of configurations suggests that the head portion alone can transduce chemical energy to mechanical motion. However, a number of ambiguities remain unresolved in these experiments, and recent work with monoclonal antibodies raises the possibility that some portion of S-2 may also be involved in tension generation.

IV. CHANGES IN PROTEIN CONFORMATION MEASURED IN SOLUTION

There is good evidence that during the powerstroke of the contractile cycle a myosin head attaches to an actin filament and exerts a force on it through a distance of approximately 10 nm. This action is certainly expected to involve rather large changes in the conformations of the two proteins, and the identification of these changes has been the goal of a number of investigations. These studies can be grouped into several classes. One, now classic set of experiments has demonstrated that the myosin molecule contains several regions of flexibility.[7,129,130] While these sites of flexibility in myosin are undoubtedly necessary for force generation, changes in the conformation of these sites are probably not primarily responsible for force generation. Some flexibility is also found in the actin polymer.[131] A second class of experiments has been concerned with the conformational changes that occur upon binding of nucleotides to myosin and to actomyosin or the changes that occur in either of the proteins upon binding to each other. The experiments that address these questions have been recently reviewed.[7,9] and the conclusions of these studies are very briefly described below.

A. Segmental Flexibility of Myosin

Some models of cross-bridge function require two sites of flexibility in the myosin molecule. If myosin heads rotate, there must be a high degree of rotational freedom in the junction between S-1 and S-2 (referred to here as the swivel). The interfilament distance in vivo varies with the sarcomere length, so that a constant volume is maintained inside the myofibril. Such variation suggests that the cross-bridges may move away from the thick filament to interact with actin. This movement would require a second site of flexibility which would logically be placed at the junction between S-2 and LMM (refered to here as the hinge). The first indication that the protein structure within these two regions may be flexible came from the pattern of proteolytically susceptible sites within myosin. Both the swivel and the hinge regions could be cleaved by a variety of enzymes suggesting a disordered protein structure.[132]

More direct evidence for flexibility within the swivel was obtained by measurements of the correlation times for Brownian rotations of myosin and its various fragments. The rotational correlation time of the myosin head was determined from the decay of anisotrophy of fluorescent probes or saturation transfer EPR spectroscopy of paramagnetic probes.[133,134] Both types of probes were bound to SH_1. The rotational correlation time for the head in HMM or myosin was significantly faster than expected if the heads were rigidly attached to the rod. An analysis of the data for S-1, HMM, and myosin led to the conclusion that S-1 was joined to the rod with almost complete flexibility. Triplet state probes attached to SH_1 have provided time-resolved measurements of the rotational motion of the myosin head.[135] Two correlation times of 0.4 and 2.6 μsec were seen which correspond to two modes of motion, one of which probably results from the rotation of S-1 relative to the rod while the other may involve motion of S-1 plus some part of S-2. Electron micrographs of myosin molecules showed that the heads made a variety of angles with the rod and that they could rotate about their long axis, again demonstrating flexibility.[53,55]

The myosin rod can be cleaved by limited proteolysis into two fragments, S-2 and LMM, and further digestion degrades the hinge region of S-2 resulting in a smaller fragment, short S-2, which lacks a region of about 200 residues in length (see Figure 1). Measurements of rotational correlation times using electrical birefringence and quasi-elastic light scattering found that both short S-2 and LMM rotated like rigid rods, but that long S-2 and rod contained regions of flexibility.[136,137] However, little evidence for flexibility within the rod was found in another study of rotational diffusion,[129] and the visco-elasticity of the rod is not consistent with a freely moving hinge.[138] These latter measurements estimated the excursion of the N-terminus of S-2 from the thick filament surface, due to large amplitude vibrational modes, to be about 25 nm. Electron micrographics of myosin molecules and filaments show that there is a flexible region in the vicinity of the junction between S-2 and LMM. Thus most studies find some flexibility within the rod but this flexibility may be due to rather restricted motions of a large region. Nuclear magnetic resonance can assess residue mobility, and the spectra of the myosin rod can limit the number of mobile residues to less than 25.[137,139] The structure of the rod will be discussed more fully later in Section IV.C where its role in the mechanism of contraction is considered.

In summary, the myosin molecule appears to possess the flexibility that is required for it to extend out from the thick filament and rotate while attached to actin.[130]

B. Conformational Changes in Myosin and Actin

A large number of investigations have sought to discover conformational changes occurring in myosin and actin in solution that may be associated with force generation. The observed conformational changes can be arbitrarily divided into two classes: global changes, i.e., those of sufficient magnitude to generate force, and local changes

which may be due to small pertubations in structure.[9] During the powerstroke, when work is performed on the thin filaments, the actomyosin complex certainly has to undergo a considerable change in conformation. To date, there is no experimental evidence that changes of the required magnitude occur in the myosin molecule in solution. There is evidence, however, that argues for considerable plasticity in the myosin head, i.e., when a perturbation occurs on one part of the molecule it often produces at least small changes in other locations. The evidence that supports these two conclusions is discussed briefly below and in more detail in References 7 and 9.

Several structural studies, discussed in Section II.B and II.D, are compatible with a myosin head that can be easily deformed. This possibility is suggested by the broad distribution of lengths, 15 to 22 nm, measured for the myosin heads of intact myosin.[53,55] Although the distribution of lengths may be an artifact of the sample preparation, it could also indicate that prior to electron microscopy a portion of the myosin can be extended during the attachment of myosin to a substrate. Electron micrographs of isolated HMM molecules bound to actin also suggested that the length of the head may be variable.[63] Electron micrographs of acto-S-1 obtained from replicas of quick-freeze deep-etch samples provide some evidence that the region of S-1 distal to actin can be deformed.[78] Subtle differences in the shape of the actoS-1 complex were induced by inclusion of negative stains during sample preparation. These differences in shape appeared to be due to changes in the region of S-1 that was distal from actin. Thus, a variety of data can be explained by the hypothesis that S-1 is easily deformed in the region proximal to the rod. This hypothesis remains unproven, but it should serve to focus attention on the possible role of this region in force generation (discussed more fully in Section V).

The shape of the myosin head in solution has been inferred from measurements of its rotational mobility and from small angle X-ray scattering, discussed in Section II.B. Addition of ATP, ADP, or PP_i to myosin, HMM or S-1 in solution caused no change in the rotational correlation time of the myosin head as measured by the decay of the fluorescence anisotropy of probes attached to the head,[140] or by saturation transfer EPR spectroscopy of attached spin probes.[134] However, these measurements may not be very sensitive to conformational changes because the myosin head has an irregular shape. It is interesting to note that all probes attached to the myosin head sense long rotational correlation times, yet it would appear unlikely that all probes are aligned along some long axis of the molecule. The irregular shape of myosin must lead to slow rotations about several axes. Thus it is possible that large deformations in shape would not cause large changes in rotational mobility. Nonetheless, these measurements are most compatible with the conclusion that nucleotides do not greatly alter the myosin shape. This conclusion is strengthened by the small angle X-ray scattering data, which find no change in the shape of S-1 upon nucleotide binding.[141] The appearance of the myosin head in electron micrographs of negatively stained myosin filaments also did not change upon addition of nucleotide.[55] We can conclude that these structural studies, with a resolution of approximately 3 nm, find no evidence for nucleotide induced changes in the shape of the myosin head.

Little change occurs in the secondary structure of myosin upon binding nucleotide. Both the optical rotatory dispersion [142] and the rate of hydrogen exchange[143] were not significantly altered by binding of ADP or ATP. Together with the structural studies cited above, these results lead to the conclusion that nucleotides do not produce global changes in the structure of the myosin head.

One of the most sensitive probes of protein conformation is the measurement of fluorescence energy transfer between two fluorophores attached to different sites on the protein. The effects of nucleotide binding on a number of distances within myosin have been measured and are discussed in References 7 and 144. Nucleotides have been

shown to have negligible effects on three distances measured within the myosin head: SH_1 to Cys177 on the A-light chain,[145] SH_1 to a reactive lysine residue in the 23 kD fragment of the S-1 heavy chain,[146] and the nucleotide site to Cys177 in the A-light chain.[147] The effect of actin on the structure of the myosin head is less well characterized than is the effect of nucleotides, however. The efficiency of energy transfer between probes on myosin was also unchanged by the binding of actin. These distances include: SH_1 to Cys177 on the light chain,[145] and SH_1 to the reactive lysine.[146] Because the sensitivity of energy transfer to a change in the distance between the chromophores is great, these negative results support the conclusion that neither nucleotides nor actin change the shape of S-1. The caveat in this conclusion is that a shape change may occur and the distances measured may not span it.

Although there is no definitive evidence for large changes in shape, there is good evidence that small changes in the conformation of myosin occur at numerous locations when nucleotides bind to the ATPase site. In a large number of the cases investigated, nucleotide binding causes a response at a second location and a pertubation at the second location affects nucleotide binding or hydrolysis. The binding of nucleotides induces a very important alteration in the structure of S-1 that decreases the affinity of S-1 for actin by a factor of 10 to 10^5.[148-150] The binding of nucleotides also has a number of other effects, including: increased tryptophan fluorescence and absorption;[151,152] changes in the mobility of probes attached to SH_1;[153,154] and the reactive lysine;[155] modification of the reactivities of SH_1 and SH_2;[156] a change in the distance between SH_1 and SH_2 (see Section C); decreased fluorescence of probes on the A-light chain;[145] and changes in the rates of cleavage of both the 20 to 50 kD and 50 to 23 kD junctions.[157-159]

The binding of actin also induces changes at multiple sites within S-1, the most important of which is the decrease in the affinity of S-1 for nucleotides.[148-150] Other effects include: alterations in the reactivities of the two thiols;[156,160] an increase in the fluorescence of an extrinsic probe attached to SH_1;[161] attenuation of the accessibility of both the 20 to 50 kD junction and the swivel to proteases;[157,162-164] and an increase in the fluorescence of a probe bound near the swivel.[165] Conversely, cleavage of the 20 to 50 kD junction decreases the affinity of S-1 for actin in the presence of nucleotides.[166]

Nuclear magnetic resonance spectroscopy of S-1 protons has suggested that actin binding has a rather dramatic effect on the internal mobility of a substantial portion of S-1.[167-169] Approximately 20% of the S-1 protons displayed sharp lines in the spectrum, indicative of motional freedom relative to the S-1 molecule. Many of these protons are located within a 41 residue N-terminal portion of the Al-light chain.[169] The spectrum of these protons was not affected by the binding of nucleotides, by proteolysis, or by immobilization of S-1. However, the binding of actin quenched this mobility, which suggests that the mobility may play some as yet undefined role in contraction.[167]

The above studies show that either nucleotide or actin induce changes in a wide variety of sites in S-1, and in fact most sites investigated are sensitive to the binding of one or both. Many of the sites affected by either actin or nucleotide are remote from their sites of binding. Several of the sites shown to be affected by actin appear to be further than 5 to 6 nm from sites on actin when measured by fluorescence energy transfer[170-172] and actin appears to affect the swivel region which is on the other end of the head.[164,165] Thus, the influence of either nucleotide or actin is felt over a large portion of the S-1 structure.[144] Although the prevalence of these effects might argue for global changes in S-1 structure, none of the effects demand more than slight perturbations in local structure.

The binding of myosin affects several sites in the actin monomer. Actin has one

reactive sulfhydryl, Cys-374. The reactivity of this group increased upon myosin binding,[156] and the florescence spectra of probes attached to the group were also altered.[173] A nucleotide binds tightly to each actin monomer and the incorporation of a fluorescent nucleotide, etheno ADP (εADP), into this site allows its properties to be probed. εADP was incorporated into actin in ghost fibers, i.e., fibers from which myosin has been extracted, and its orientation was monitored by measuring the polarization of its fluorescence. The binding of S-1 or HMM caused neglible changes in the orientation of the nucleotide.[174] However, when both myosin and ATP were added to the fiber the average angles of the nucleotide were unchanged but the spread in orientations increased.[175] F-actin in muscle fibers can be labeled with a fluorescent derivative of the fungal toxin phalloidin, whose fluorescence polarization was also altered during active generation of tension.[176] These results indicate that myosin affects actin conformation in the vicinity of the probes but they do not necessarily require large changes in structure. The possibility that interaction with myosin alters the helical array of actin monomers in F-actin has also been investigated. Quasi-elastic scattering of light has shown that the elastic modulus for bending of the polymer was decreased by the binding of HMM.[177] However, the efficiency of fluorescence energy transfer between probes within a monomer and between probes on different monomers was not changed by binding S-1.[178] The scattering data show that formation of the actin-myosin bond alters actin-actin interactions within the polymer, however, the energy transfer suggests that this does not involve a change in the helical array of monomers. In summary, the interaction of myosin with actin produces changes in actin structure at numerous sites, but the observed changes give little clue to the function of actin in force generation.

Actin and myosin subfragments form a tight complex with a well-defined structure. Although addition of nucleotides weakens this interaction, the ternary complexes with ADP and AMPPNP can be achieved fairly easily in solution. Some hypotheses for the force-generating mechanism have postulated that tight coupling should occur between the nucleotide bound to myosin and the orientation of the myosin head.[7,144] To investigate this possibility, the effect of ADP or AMPPNP on the orientation of the myosin head was monitored by measuring the efficiency of fluorescent energy transfer between probes on myosin (SH_1) and on actin (Cys-374). The efficiency was not changed by the binding of either nucleotide.[171] This result supports conclusions drawn from studies of paramagnetic probes, which indicate that SH_1 resides in a domain of myosin whose orientation relative to actin is not sensitive to the events which produce force, discussed in Section V.

Although it is expected that large changes in protein structure should accompany the contractile interaction, the experiments cited above have failed to find such changes in the global structures of myosin or actin during the interactions studied in solution. The lack of evidence for large changes in configuration could be due to several possibilities. Large changes could have occurred but not been detected by the methods used. Alternatively, the states that generate force in muscle fibers may not be present in significant fractions in these solution studies.

C. Studies of Protein Conformation that may Suggest Mechanisms for Contraction

Some of the results cited in the previous section have lead to the conclusion that many small changes occur in the structures of actin and myosin during their interactions; however, none of the results were directly interpreted in terms of possible involvement in a contractile mechanism. There have been several experimental findings that have suggested possible mechanisms for contraction. First, the observation that nucleotides cause a substantial shift in the distance between two thiols could indicate movements of domains within myosin. Second, myosin has been shown to have two interconvertible conformations and the transition between them has been postulated to

be involved in the generation of force. Third, a helix to random coil transition in a portion of S-2 could shorten the length of this link and pull a rigid S-1 through a 10 nm powerstroke. And finally, two recent results have suggested that the orientation of S-1 changes relative to actin. These observations and the conclusions that can be drawn from them are discussed below.

Bifunctional reagents that cross-link SH_1 to SH_2 have shown that the distance between these residues decreased when nucleotides bound to the ATPase site.[179,180] The distance between the sulfhydryls varied from a maximum of 1.4 nm in the absence of nucleotides to a lower limit of 0.2 nm in the presence of nucleotides.[181,182] Cross-linking of the sulfhydryls in the presence of nucleotides and divalent cations resulted in the trapping of both the nucleotide and cation at the active site.[183] The release of these ligands was several orders of magnitude slower than in the native molecule. The use of fluorescent energy transfer to measure changes in the distance between probes on the two sulfhydryls produced conflicting results. One study found 2.8 nm in the absence of nucleotides, decreasing to 2.1 to 2.2 nm in the presence of nucleotides.[184] A second study found a distance of 4.6 nm and no effect of nucleotides.[185] These studies are complicated by the fact that the bulky probes used to measure the distance are large compared to the expected distance between the two sulfhydryls. Taken together the results show that nucleotide binding produces a change in the conformation of myosin in the vicinity of the two sulfhydryls, a change which entraps the nucleotide and its accompanying cation within the structure of the protein. As discussed in the previous section, the nucleotide site is 2 to 4 nm from the sulfhydryls so that these bidirectional effects are transmitted over a considerable distance. Furthermore, cross-linking the two thiols changes the conformation of myosin so that its affinity for actin is the same as that measured when ATP or ADP·P_i are bound to the myosin ATPase site, discussed in section VI. This observation shows that this cross-link induces functionally significant changes in the conformation of myosin. However, small angle X-ray scattering does not detect a large change in the overall shape of the myosin head due to cross-linking of SH_1 and SH_2, thus the change in conformation may also be local.[359] Other enzymes are known to trap substrates within clefts formed by protein domains to enable the action of the protein on the substrate to be carried out surrounded by protein groups in a largely nonaqueous medium.[186] In the case of myosin, the trapping of the nucleotide could have an additional function. If the products of hydrolysis remain bound to the myosin during the powerstroke they would prevent dissociation of the head by ATP during the powerstroke resulting in more efficient energy transduction.

Evidence that the myosin head exists in two different interconvertible forms has been provided by nuclear magnetic resonance of bound nucleotides and ^{19}F attached to the SH_1.[188-191] In all cases the spectra showed two peaks which could be assigned to two different myosin conformations. The populations of the two forms were functions of both the temperature and the fraction of myosin with bound nucleotide. Of particular importance are the magnitudes of ΔH, 15 kcal/mol, and ΔS, 55 cal/deg-mol, for the transition between the states in the presence of nucleotide. Both these values are large and suggest that an extensive change in protein structure accompanies the transition. Some effort has been made to correlate these two states with intermediates observed during the hydrolysis of ATP by myosin.[191]

In particular, one state has been identified as existing at the beginning of the powerstroke while the second state is thought to represent the end of the powerstroke. The identification, however, is problematic. The nature of the states at the beginning and end of the powerstroke are likely to be strongly dependent on the details of the bond between actin and myosin and to date these two conformations have only been observed in myosin by itself. Secondly, while the magnitudes of ΔH and ΔS are large,

the magnitude of ΔG is close to zero at room temperature. Since it is the value of ΔG which will determine the amount of work that can be done, it is not clear how work could be derived from a transition between these states. Nonetheless, these observations are important in that they show that myosin exists in two states which probably play an important role in the kinetics of ATP hydrolysis, and possibly also in the generation of force.

One model has been proposed in which force is generated by a conformational change in S-2 rather than in S-1.[25,192] Force generation is thought to involve a transition of a region of S-2 from a helix to random coil configuration. The length of the polypeptide is shorter in the random coil than in the helix and the melting of approximately 165 residues per strand will shorten S-2 by 10 nm, the estimated length of the powerstroke. This model was first suggested by the observation that a region of S-2 appeared to have a lower thermal stability than the rest of the rod. During very limited proteolysis the rod is clipped in a well-defined region to produce two fragments, long S-2 and LMM. More extensive digestion produced multiple additional cuts that shortened the long S-2 about one third of its length.[193] The region removed by digestion is labeled as the hinge in Figure 1. As discussed earlier, some studies have suggested that this region is flexible, a result that is contradicted by other studies. A region of lower thermal stability exists in the myosin rod and in long S-2 and it is likely that it corresponds to the region of high proteolytic susceptibility.[194] Temperature-jump measurements showed that the transitions associated with this instability in structure can occur with sufficient speed, in the submillisecond range, to play a role in tension generation.[195] However, more recent work using differential scanning calorimetry has not found an extensive region that would be partially melted at physiological conditions.[196] thus, there is considerable uncertainty concerning both the flexibility and the stability of this region.

In the model proposed by Harrington et al.[192] the force producing helix to coil transition is triggered by the release of S-2 from the filament backbone. The use of cross-linking agents has shown that S-2 could be cross-linked to the filament core in both myosin filaments and in rigor myofibrils. In the absence of divalent cations the degree of cross-linking was highly pH dependent and decreased dramatically as the pH was raised from 7.0 to 8.4;[25] however, in the presence of divalent cations this decrease was not seen so that its occurrence under physiological conditions remains hypothetical.[197]

Generation of force by a helix to coil transition in S-2 is the only hypothesis for force generation which identifies a molecular mechanism. However, there are a number of problems with this hypothesis. There is no direct evidence that the transition occurs during muscle contraction, or that a movement of S-2 from a site on the filament core is required for contraction. A more troublesome difficulty is the mechanochemical transduction of energy in systems where no myosin filaments are present. However, the possibility that some shortening occurs in S-2 during the powerstroke is not excluded completely, and the region of S-2 adjacent to the head may be involved in force generation, as suggested by the inhibition of function caused by antibodies which bind to it.[126]

It is possible that some region of S-2 forms the passive elasticity that has been shown to exist in the cross-bridge by measurements of fiber mechanics. It should be noted that a coiled-coil structure itself is sufficiently rigid that S-2 would not contribute to cross-bridge elasticity unless it contains unstructured regions.[33,138] However, cross-linking of S-2 to the filament backbone has been shown to have only a small effect on the stiffness of rigor fibers so that it appears unlikely that S-2 plays a role in cross-bridge elasticity.[198]

Two recent results suggest that a substantial region of S-1 can change its orientation relative to the actin filament. The structure of the actoS-1 complex in the presence of

ATP was examined by electron microscopy after first cross-linking S-1 to actin using the zero-length cross-linker discussed earlier.[199] The orientation of single S-1 molecules could be resolved. In the absence of ATP, S-1 was curved and appeared to make an angle of 45° with the thin filament as also observed for the uncross-linked complex. However, during ATP hydrolysis, S-1 molecules were attached at variable angles and appeared both shorter and fatter. Further evidence for movement of S-1 relative to actin was obtained by measurements of fluorescence energy transfer between a donor on cys-177 of the A-light chain and an acceptor on cys-374 of actin.[200] In the absence of actin these two sites were approximately 6 nm distant. When SH_1 was cross-linked to SH_2, or during the hydrolysis of ATP, this distance decreased to less than 3 nm. This is in contrast to the results obtained with probes on SH_1, whose distance from cys-374 was unchanged by nucleotides.[171] Cys-177 of the light chain is located in the half of S-1 that is distal from actin,[201] thus these results suggest a substantial movement of this region.

Both the above results suggest that S-1 may generate force by changing its orientation; however, both experimental approaches have unresolved ambiguities. It is expected that the affinity of S-1 and actin becomes very low at some point in the ATPase cycle, and the possibility that even the cross-linked species can dissociate has not been eliminated. Such dissociation has been suggested by the preliminary observation of msec mobility of a spin-label attached to the myosin head during the hydrolysis of ATP by cross-linked actoS-1.[202] A determination of the amount of energy transferred between probes on myosin and on actin required an accurate estimate of the fraction of S-1 bound to actin. Although this fraction was measured for some cases, it was calculated for others. Because the affinity of these proteins is somewhat uncertain, these calculations introduce considerable uncertainty into the estimation of the distances between probes. Although the problems raised above preclude a definitive conclusion, these results nevertheless do lend support to the hypothesis that a change in S-1 orientation occurs during ATP hydrolysis.

V. CROSS-BRIDGE CONFORMATION IN FIBERS

A. Rigor Fibers

Rigor fibers are a stable and easily studied system, in which the cross-bridges are most probably found in several configurations that are the product of two forces, the constraints of the filament lattice and the energetics of the strong bond between actin and myosin. Because the helical periodicity of the actin and myosin filaments do not match one another, considerable distortion is required to obtain a specific actomyosin interaction. This distortion could occur in the contractile or elastic elements known to exist within the cross-bridge or in the helical arrangement of the thin filament. Upon removal of ATP, fibers go into rigor and develop isometric forces that can be as large as those developed during the active state and which scale with filament overlap.[203,204] Although high forces can be attained, these tension-generating states appear quite different from those of the active cycle. Following a step decrease in length, a limited recovery of tension occurs with a time course that is several orders of magnitude slower than that of active fibers.[205] An analysis of this slow recovery led to the suggestion that in rigor muscle cross-bridges bind to actin in several states with very slow transitions between them.[206]

Electron micrographs of thin sections of rigor insect flight muscle formed part of the evidence which first suggested that force production by myosin cross-bridges may involve a change in their orientation.[207] The axis of the cross-bridge appeared to make an acute angle with respect to the filament axis in rigor while relaxation produced a

more perpendicular orientation.[208] In thin sections, pairs of cross-bridges were observed to interact with each actin filament with a periodicity of 38 nm, equivalent to the periodicity of the helix of the actin filament. These structures were dubbed double chevrons due to their distinctive appearance (some such pairs of angled bridges are shown in the upper right hand corner of Figure 6). Using X-ray diffraction, the effects of sample preparation on cross-bridge structures has been shown to be slight.[209] Diffraction of electron micrographs of rigor muscle show doublets of cross-bridges, the leading one well-defined and sharply angled, and the trailing one more diffuse and perpendicular.[210] More recently, insect flight muscle has been visualized using a freeze etch technique that does not involve traditional fixation and staining.[78] Pictures produced by this technique, shown in Figure 6, also showed angled cross-bridges, however, the angles were less acute. The orientation of individual cross-bridges was measured directly to give an angular distribution, extending from about 90 to 50°. The variation in orientations, which can be seen in Figure 6, may be due to the lattice constraints or to an artifact, and a distribution of similar breadth can also be seen in the positively stained thin sections.

The structure of rigor insect flight muscle has recently been obtained at higher resolution, 4.6 nm, by diffraction and image reconstruction of electron micrographs of tilted thin sections.[211] Cross-bridges extended from the surface of the thick filament to their attachment sites on the thin filament in the characteristic double chevron pattern of rigor muscle. The cross-bridges were 17 to 19 nm in length, comparable to the length of the myosin head measured by other techniques. The axial dimension of the cross-bridge was 11 nm which is larger than expected if each cross-bridge is formed by one myosin head and is sufficient to contain two myosin heads. The bond between actin and the myosin head appeared similar to that observed in the actoS-1 complex,[58] with the myosin head binding to actin in the crevice between the strands. As discussed above, the mismatch between the thick and the thin filament lattice periodicities requires sites of flexibility, and several sites were evident in the reconstructions. While the lead cross-bridge of the chevron was angled axially by about 50°, the rear cross-bridge appeared to have a more perpendicular orientation with an attachment angle of 78°. In addition, the slew of the lead cross-bridge around the thin filament was opposite to that observed in reconstructions of actoS-1 complexes. The difference in orientations and the pronounced departure from the actoS-1 structure was most prominent in that portion of the head that is distal from actin. Spectra of fluorescent and paramagnetic probes suggest that a portion of myosin adjacent to actin is well-ordered in rigor fibers, and little change in this region was found in the reconstructed images. Another site of flexibility was found in the actin polymer. The actin filament was twisted around its axis and the two strands were separated by their interaction with myosin. As discussed in Section II.C, the pitch of the actin helix also appeared variable in electron micrographs of pure actin. The separation of the strands was unexpected and may be an artifact of the preparation.

X-ray diffraction has provided a powerful tool for studying the structure of muscle fibers. In vertebrate muscle the patterns contain two sets of layer lines, one arising from the helical array of myosin heads around the thick filament based on orders of 43 nm, and one arising from the helical array of actin monomers in the thin filament based on orders of 72 nm.[1,13-15] In relaxed muscle the scattering from the myosin filament is strong showing the myosin heads are associated with the thick filament. In rigor muscle the thick filament based layer lines are absent, and the intensities of the actin layer lines are greatly enhanced. The distribution of intensity along the layer lines is governed by the reciprocal relationship characteristic of diffraction patterns so that intensity closer to the meridian is produced by mass at a larger radius. The additional intensity of the actin layer lines in rigor muscle is, in general, found closer to the

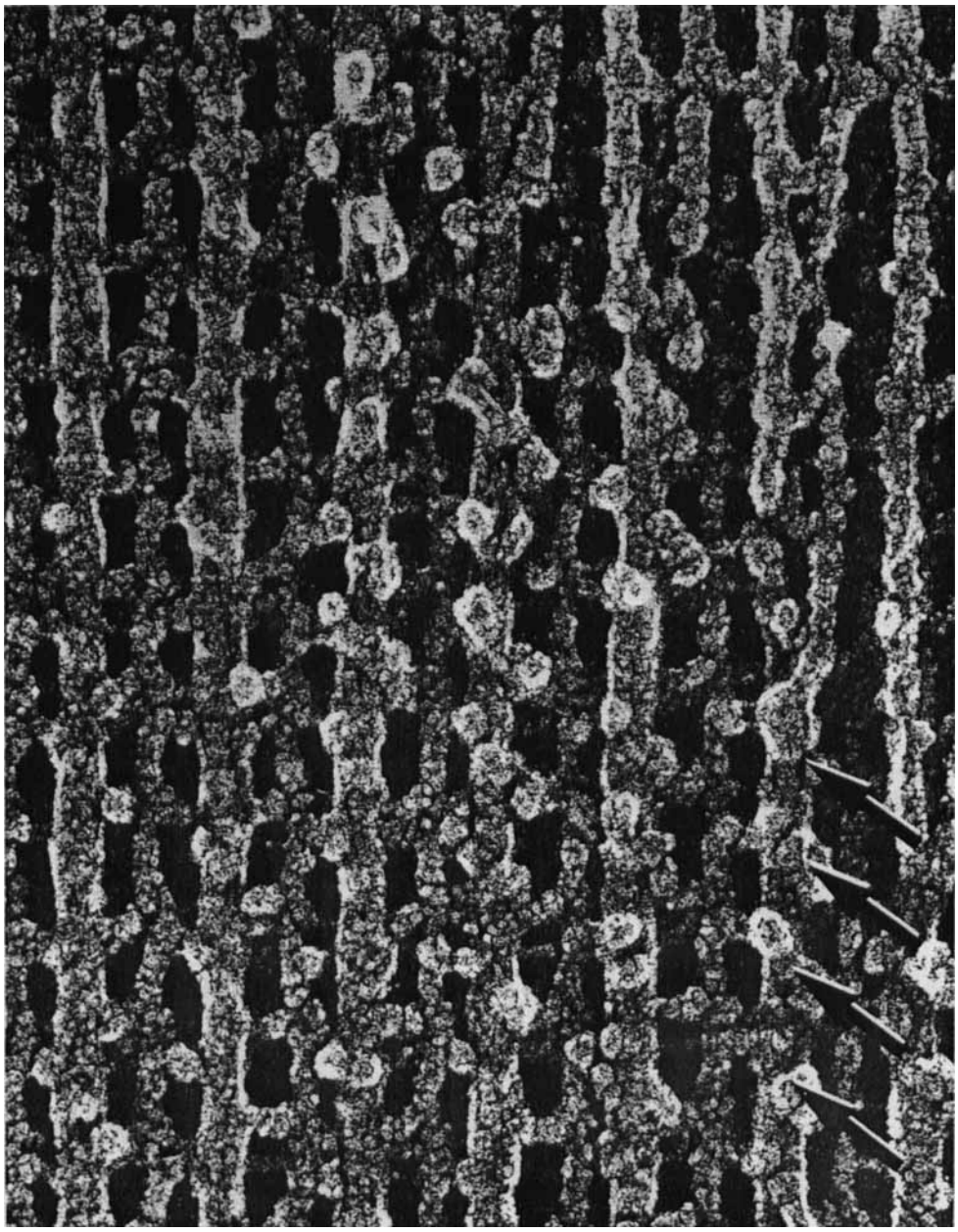

FIGURE 6. Electron micrograph of rigor insect flight muscle. The sample was fixed with aldehyde, frozen quickly, freeze-etched and plated with platinum. On the far left the plane of the fracture has passed through the "myac layer" defined by Reedy.[208] Cross-bridges can be seen to form a variety of angles with the filament axis. There is some polarity to these angles which is more easily visualized by sighting along the axis of the filaments (the Z line is out of the picture towards the bottom). At the upper left can be seen several examples of the double chevron pattern, a pattern which is more evident in negatively stained thin sections. In the center of the micrograph the plane of the fracture lifted above the "myac layer", revealing the two start left-handed helical arrays of cross-bridges on the thick filaments (in register with the black arrows). (Magnification × 398,000.) (From Heuser, J. and Cooke, R., *J. Mol. Biol.*, 169, 97, 1983. With permission.)

meridian than that observed from the actin helix alone in relaxed muscle. This is to be expected if the increase in the intensity of the actin layer lines is due to the binding of

myosin heads to the surface of the actin filament. Considerable effort has gone into modeling the observed X-ray diffraction patterns of rigor insect flight muscle.[212-214] A simple shape was assumed for the myosin head, the fraction of myosin heads attached to actin was estimated, and the expected intensity distributions along the actin based layer lines were calculated as the axial and azimuthal orientations of the attached heads were varied. The best fits to the data were obtained when the axial and azimuthal angles were both about 45°, i.e., in an orientation similar to that seen in reconstructions of the actoS-1 structure. The fit of model to experimental data was quite good. These models assumed that a significant portion of the myosin heads were unattached in rigor. It is probable that more information could be obtained using a higher occupancy of cross-bridge attachment positions and newer more realistic shapes of the myosin-head as recently determined from the actoS-1 complex.

The interpretation of the X-ray diffraction patterns discussed above assumed that the myosin heads, when bound to actin, formed a well-ordered array. However, electron micrographs have shown that there is some disorder to this array. The effect of disorder will be to diminish the intensity of the layer lines and to increase the amount of diffuse X-ray scattering seen near the origin in the diffraction pattern. In fact, diffuse scattering from rigor muscle has been observed and attributed to disorder in the cross-bridges.[215] The scattering was more intense along the equator showing that the disorder was more pronounced azimuthally than axially.

Both the orientation and motions of myosin heads in glycerinated muscle fibers have been monitored using fluorescent and paramagnetic probes attached to the head. Several fluorescent probes attached to SH_1 were highly ordered with respect to the fiber axis.[216-218] The spectra of paramagnetic probes attached to the SH_1 also showed that the probes all had approximately the same orientation. The distribution of probe angles could be determined from the spectra and was approximated by a Gaussian with a full width at half maximum of 10 to 15°.[219] Both fluorescent and paramagnetic probes can be used to measure molecular rotations, and both probes showed that the myosin head was effectively rigid in rigor muscle.[220,221] Saturation transfer EPR spectra, which are sensitive to slow rotations, showed that the rotational correlation times of the paramagnetic probes were slower than 1 msec.[221]

All the results obtained with probes attached to the reactive sulfhydryl lead to the same conclusion: this portion of myosin is rigidly attached to actin with a specific and well-defined orientation. This orientation is determined by the interaction with actin, and if this interaction is removed, by relaxing the fibers or by stretching to eliminate filament overlap, the spectra of the probes change dramatically to one characteristic of a highly disordered angular distribution. In rigor fibers from rabbit psoas muscle there was no detectable disordered component in the spectra of paramagnetic probes showing that the fraction of myosin heads not attached to actin is small, less than 5%.[207] In insect flight muscle there was a small disordered component in the spectrum which could arise from myosin heads that are not specifically attached to actin or, more likely, could arise from probes that are not attached to SH_1.[222] These spectra place an upper limit of about 20% on the fraction of myosin heads that are not attached to actin in rigor insect flight muscle.

Two enzymatic techniques have also shown that all myosin heads are attached to actin in rigor vertebrate muscle. In the absence of divalent cations myosin has a high ATPase activity which is inhibited by the binding of actin. The ATPase activity of fibers showed that more than 95% of the heads were bound to actin in glycerinated rabbit muscle.[223] Binding to actin blocks the digestion of myosin at the junction between the 20 kD and 50 kD fragments. An analysis of the digestion of rigor myofibrils demonstrated that all myosin heads were attached to actin in vertebrate muscle but that only about 80% were attached in insect flight muscle.[224] This estimate should again be

considered as a lower limit on the fraction attached, since weakly bound heads, or heads bound in strained configurations may have different patterns of digestion.

The stoichiometry of myosin molecules and cross-bridges is also consistent with a large fraction of attached heads. In insect flight muscle there appears to be four myosin molecules for each 14.5 nm repeat of the thick filament[225] and there are four cross-bridges observed in a "flared X" pattern at each repeat.[207] Electron micrographs obtained from rapidly frozen fibers and reconstructions of thin sections both show cross-bridges that are larger than a single myosin head and are approximately the size expected for two heads. Both techniques should have been able to observe unattached heads, but none were found.[78,210,211] Together, the data suggest that each cross-bridge observed in electron micrographs of rigor insect flight muscle consists of the two heads of one myosin molecule and that to a first approximation there are eight attached heads and four cross-bridges at each 14.5 nm of the thick filament repeat.

Myosin molecules contain sufficient flexibility for each head to attach to a different actin filament in solution[226] and in fibers.[227] The possibility of such binding in rigor fibers has been discussed in detail,[228] however, it appears that in the majority of cases the two heads of each myosin are bound to the same actin filament.[211]

The presence of an elastic element within the cross-bridge has been suggested by the response of active muscle to step changes in length, discussed in more detail in Section V.D. This elastic element is also present in rigor fibers,[198,229] Its location has been investigated in experiments where stress is applied to rigor fibers. The orientations of paramagnetic probes attached to SH_1 and a fluorescent nucleotide bound to the ATP-ase site on myosin, were unchanged by stress, showing that the bond between actin and myosin is rigid.[230,231] The ratio of the equatorial reflections in the small angle X-ray diffraction pattern was also unchanged by stress, showing that stress does not induce a motion of the heads that would cause a radial redistribution of mass.[232] The polarization of tryptophan fluorescence was constant as the length of rigor fibers was oscillated.[233] Because a large fraction of heads are attached in rigor fibers while a smaller fraction are attached in active fibers, the stress per cross-bridge in the above experiments may be lower than that of active cross-bridges. To circumvent this problem, fibers with low filament overlap were stretched, but again no change in orientation of paramagnetic probes attached to SH_1 was detected.[234] All of the above results suggest that myosin heads do not move upon the application of stress, and therefore the cross-bridge compliance might be located in the S-2 fragment. However, cross-linking S-2 to the thick filament backbone has been shown to have only a small effect on the stiffness of rigor fibers, indicating that the compliant element is not found in S-2.[198] One hypothesis consistent with the above observations is that the cross-bridge compliance lies in the myosin head between S-2 and the region containing SH_1 and the nucleotide binding site. Distance measurements using fluorescence energy transfer have shown that neither SH_1 nor the nucleotide binding site is closer than 5 nm to other probe sites on actin.[170,172] These distances would exclude the two myosin probe sites from a region that comprises approximately one third to one half of the myosin head proximal to actin. Thus, the compliant element is most probably found in the half of the myosin head that is distal from actin — the same region which appears to show flexibility in the reconstructions of electron micrographs of rigor muscle and in electron micrographs of HMM bound to actin.[63,211] Stress induced movements of this region would have to be such that they did not greatly alter the radial mass distribution or the net orientation of tryptophane residues.

B. Relaxed Fibers and the Structure of the Thick Filament

Relaxed muscle has a very low stiffness and is readily extensible, suggesting that

cross-bridges either do not interact with the thin filaments or that their interactions are weak and/or transitory. Evidence that cross-bridges are not interacting strongly with actin has been obtained from electron micrographs, X-ray diffraction patterns, and from measurements of the motions and orientations of attached probes. In spite of the fact that relaxation represents a stable physiological state and therefore is amenable to investigation, the exact configuration of the cross-bridges in this state remains controversial.

The early structural studies of muscle regulation suggested that the actomyosin interaction may be stericaly blocked by tropomyosin.[80] Subsequent structural studies have questioned this view and the validity of this model remains controversial.[235,236] The question relevant to the present discussion of cross-bridge action is whether myosin heads attach to actin in relaxed muscle, and if so, what is the nature of the attachment? Recent studies done in solution at low ionic strength have shown that troponin and tropomyosin effectively blocked the actomyosin ATPase activity in the absence of calcium, but that actomyosin binding still occurred.[235] Under similar conditions the stiffness of relaxed glycerinated fibers was low in slow stretches but high in fast stretches.[236] These data suggest that at low ionic strength some cross-bridges are attached to actin filaments but that the attachment is rapidly reversible. At physiological ionic strength the stiffness of relaxed fibers is much lower than observed at low ionic strength and thus any binding of actin and myosin that does occur in vivo must be either very weak or the lifetime of the attached state must be short, approximately less than 10^{-4} sec. However, the observation that actomyosin binding occurs in fully relaxed fibers at low ionic strength suggests that it probably also occurs to at least some extent under physiological conditions.

Small angle X-ray diffraction patterns show that in relaxed muscle the myosin cross-bridges no longer enhance the actin layer lines but take up positions characteristic of a helical array around the thick filaments.[1-3] In vertebrate skeletal muscle, three myosin molecules project radially from the filament at intervals of 14.3 nm.[225] The angular twist between triplets produces a helical repeat at every 42.9 nm, giving rise to a series of layer lines indexing on 42.9 nm. As first described by Squire, the helical arrays of filaments from different types of muscle are related to one another.[237] As in the case for rigor muscle, one can attempt to determine the cross-bridge structure in relaxed muscle by assuming a shape and orientation for the myosin heads, calculating the expected intensities along the layer lines and then comparing these expected intensities to those observed. Such simulations have shown that the myosin heads in relaxed vertebrate muscle do not project at right angles to the surface of the thick filament but are skewed around and along it.[237,238] The strong intensity of the 42.9 nm layer line indicates that the cross-bridges line up approximately along their helical contours around the thick filament. The best fits to the data of vertebrate skeletal muscle were obtained when the two myosin heads of each cross-bridge were considered to tilt in opposite directions axially by 20 to 40° from the normal and were skewed around the filament so that they lay roughly on a tangent to the filament surface. The center of mass of each cross-bridge is about 3 nm from the surface of the filament backbone, which has a radius of about 5 to 6 nm. Because the distance from the surface of the thick filament to that of the thin filament is of the order of 20 nm at rest length, the myosin heads in this model did not approach closely to the thin filaments. Similar patterns are found in some relaxed invertebrate muscles, while in others the cross-bridges appear to be more normal to the filament axis.[239]

The cross-bridge array of the thick filament has also been investigated by an image analysis of electron micrographs of negatively stained thick filaments.[240-243] Reconstructions of filaments isolated from Limulus, shown in Figure 7, revealed elongated projections that are thought to represent unresolved pairs of myosin heads. These pro-

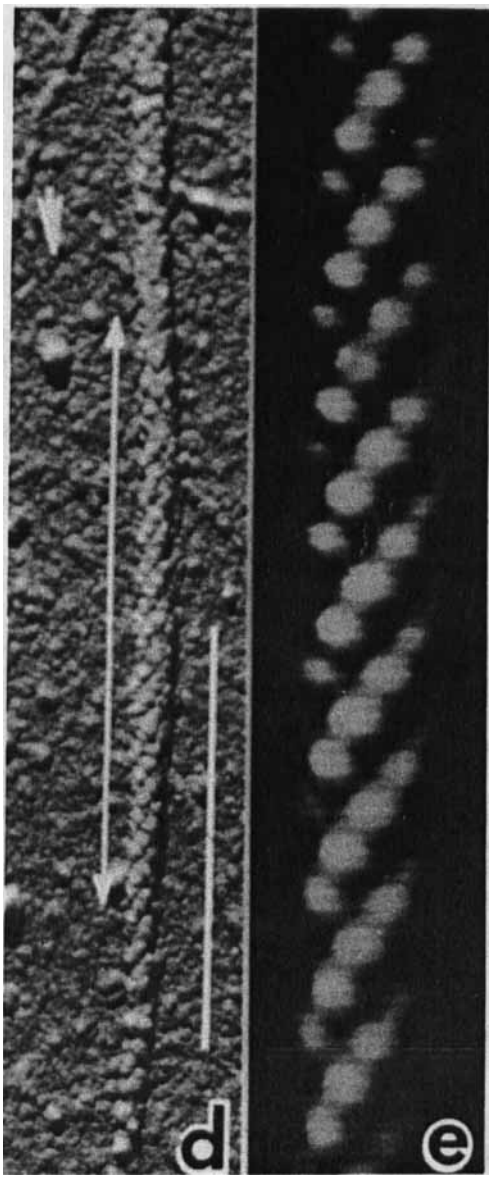

FIGURE 7. Myosin filaments isolated from *Limulus* muscle. An electron micrograph of a platinum shadowed filament is shown on the left (d). The arrow shows the direction of shadowing. (Magnification x 109,400.) An optically filtered image of the region indicated by the double arrow is shown on the right (e). The cross-bridge projections form integral surface helices, with an axial spacing of 14.5 nm between adjacent crowns and a major axial repeat every 43.5 nm. The array of cross-bridges form a four start right-handed helix. (From Levine, R. J. C., Kensler, R. W., Reedy, M., Hoffman, W., Davidheiser, S., and Davies, R. E., *Contractile Mechanisms in Muscle,* Pollack, G. H. and Sugi, H., Eds., Plenum Press, New York, 1984. 93. With permission).

bridges around the thick filament has dropped by 50%, there is now more mass ordered on the basic cross-bridge repeat of 14.3 nm. As was discussed earlier, there is probably considerable cross-bridge disorder in relaxed muscle and it is plausible that some aspects of the cross-bridge array become more ordered in contracting muscle. One likely explanation put forward by these investigators is that force generation has straightened out, and aligned S-2 and adjacent portions of S-1 such that they now line up more precisely with their thick filament origins.[13]

Fluorescent extrinsic and intrinsic probes have both been used to measure the orientations and motions of the myosin heads in contracting fibers. Different cross-bridge orientations were found in contracting, relaxed, and rigor muscle by measurements of the polarization of tryptophane fluorescence.[233] Results obtained with fluorescent probes attached to SH_1 have been mixed. Early studies found no difference between relaxed and contracting muscle,[276] but more recent studies found that the linear dichroism of these probes is anisotropic in contracting muscle, showing that some cross-bridge order is present.[245] The anisotropy in contracting fibers resembled that found in the presence of ADP and was different from that found in rigor. Thus, the predominant attached states during contraction have bound nucleotides, a result also found by measuring the spectra of paramagnetic or fluorescent probes that sense nucleotide binding.[277,278]

Cross-bridge cycling rates in glycerinated psoas are approximately two S^{-1} which is much slower than the rotational correlation times that can be measured by steady state techniques. A study of the fluctuations in orientation of fluorescent probes attached to SH_1 was able to analyze motions in this time domain and found that slow changes in orientation did occur, showing that cross-bridges do change orientation during the contractile cycle.[279] Whether these changes in orientation occur while myosin is attached to actin remains unresolved.

Paramagnetic probes have suggested that the region of myosin in the vicinity of SH_1 does not change its orientation during the powerstroke.[262,280] The positions of the three hyperfine lines in the EPR spectrum depend on the orientation of the probe axes relative to the applied magnetic field, allowing one to determine the angular distribution of the probes with a resolution of 1 to 2°. The spectra of probes attached to the SH_1 of glycerinated rabbit psoas fibers were found to be a linear superposition of 20% of the spectrum of rigor muscle and 80% of the spectrum of relaxed muscle. The size of the disordered fraction decreased in a hyperbolic fashion as the ATP concentration was raised extrapolating to approximately 80% as the substrate concentration approached infinity, showing that the fraction of probes that were ordered did not arise due to insufficient substrate. In a preliminary report it was found that saturation transfer EPR spectra could also be decomposed into two fractions: a mobile fraction, with a rotational correlation time which resembled that of heads not interacting with actin, as seen in relaxed fibers; and an immobile fraction with a rotational correlation time which resembled that found for rigor fibers.[281] The fractions of the mobile and immobile probes corresponded to the fractions of ordered and disordered probes, respectively. Thus 80% of the probes were disordered and executing Brownian rotations in times of 1 to 10 μsec, with both EPR and saturation transfer EPR spectra that were indistinguishable from those of relaxed muscle. This strongly suggests that these probes are on heads that are detached from actin. The remaining 20% of the heads appear to be attached to actin with probes oriented as in the rigor state. Although the probes have the same orientation as in rigor, this should not be taken to mean that the head is also devoid of nucleotide since these paramagnetic probes do not sense the presence of nucleotides.

The orientation of a fluorescent nucleotide, ethenoATP, was also highly ordered in contracting muscle.[231,247] At low nucleotide concentration the orientation of the nu-

jections were almost parallel to the filament surface and lay along the right-handed helical tracks of pitch 43 nm. Recently, the two heads have been resolved in relaxed thick filaments from tarantula muscle.[241] Images of filaments isolated from frog muscle showed that the myosin molecules were arranged in a triple-stranded helical array.[243] The reconstructions derived from the electron micrographs of isolated thick filaments are similar to the structures predicted from the X-ray diffraction patterns of relaxed muscle described above.

Electron micrographs of thin sections of relaxed insect flight muscle showed cross-bridges that were approximately perpendicular to the fiber axis.[207-210] Filtered images of such thin sections showed mass projecting almost straight out from the thick filament at intervals of 14.5 nm. The connection between this mass and the thin filament appeared more tenuous than that observed in rigor muscle. These micrographs thus lead to a picture in which cross-bridges extend out at right angles to the thick filament and interact to some extent with the thin filaments. This picture is very different from the tilted skewed cross-bridges adjacent to the thick filament found from modeling the small angle X-ray diffraction patterns and from reconstructions of isolated thick filaments. The differences in these two views may be due to limitations in the techniques. Myosin heads that are detached from actin may not be clearly resolved in the negatively stained thin sections. The fixation used to prepare thin sections may trap myosin heads that attach transiently to actin. X-ray diffraction patterns obtained before and during these procedures show some enhancement of actin based diffraction after fixation suggesting that some trapping of myosin heads on actin may occur.[209] On the other hand diffraction is only sensitive to ordered arrays and therefore any disordered myosin heads will be missed.

Visualization of relaxed insect flight muscle by a rapid freezing technique showed a different picture from that obtained by positive staining.[78] Here the surface of the thick filament was covered with globular structures that probably represent myosin heads detached from actin. However, the helical array of the cross-bridges had been lost during the preparation of the sample. These micrographs of relaxed fibers also showed some cross-bridges attached to actin, with a broad distribution of orientations centered at 90° to the filament axis. More recently, similar methods have been able to visualize the helical array of the myosin heads in relaxed vertebrate fibers.[244] These showed a three start right-handed helical array of cross-bridges that extended radially to 15 nm. A similar radial extent was predicted from the small angle X-ray diffraction studies discussed above.

In summary, electron microscopy of relaxed fibers has suggested two configurations for myosin heads, one in which they are attached to thin filaments in a variety of orientations centered around the perpendicular, and one in which they form helical arrays about the thick filament similar to those deduced from X-ray diffraction and from reconstructions of isolated thick filaments.

In sharp contrast to the ordered arrays of myosin cross-bridges discussed above, the spectra of probes attached to the myosin head in relaxed fibers showed extensive orientational disorder and motion on a μsec time scale. The anisotropy of both the absorption and the emission of fluorescent probes attached to the SH_1 site was much less than that in rigor, indicating more disorder in the probes.[217,245] Disorder, however, was not complete, and some degree of orientation that was independent of filament overlap has recently been found.[246] Similar disorder was seen with fluorescent nucleotides bound to myosin.[247] The angular distribution of paramagnetic probes attached to SH_1 was indistinguishable from isotropic in both vertebrate and insect flight muscle.[219,222] An analysis of the spectra of fluorescent probes on SH_1 shows that the probe orientations are not completely random, and can be described by a Gaussian distribution with a full width at half height of about 60°. The mean angles of the probe distributions were similar in rigor and relaxation.[248]

Two spectroscopic methods that are sensitive to long rotational correlation times have both shown that probes attached to SH_1 execute large angle Brownian rotations in the microsecond time range in relaxed fibers. Saturation transfer EPR spectra of paramagnetic probes found a correlation time of 1 to 10 μsec.[221,249,250] The phosphorescence of triplet state probes has a long lifetime and allows one to resolve multiple rotational modes in the time range of microseconds to milliseconds. When one such probe, eosin, was attached to SH_1 in synthetic myosin filaments it was found to rotate with two correlation times, a fast one of 700 nsec and a slow one of 5 μsec.[135] The anisotropy did not decay to zero within 500 μsec showing that the probe distributions were not randomized completely. Thus, either the motions of all probes are restricted in amplitude or some fraction of probes do not rotate at all.

All of the above measurements agree that probes bound to myosin heads are highly disordered and mobile in relaxed fibers and myosin filaments. The disagreement between this view and the ordered arrays seen in electron micrographs and X-ray diffraction patterns could have several explanations. An unresolved question concerns the presence of order in the preparations examined in the studies using probes. Glycerinated rabbit muscle has been extensively used in the probe studies and it displays a weaker diffraction pattern than found for other muscles.[251] Furthermore, the presence of a myosin-based diffraction pattern in the labeled fibers or filaments from rabbit muscle has not yet been checked. The ordered array may have been lost during the reaction with the probes, a possibility now under investigation. An alternative explanation is that the two sets of data are not incompatible. Both orientational disorder and rotational motion can be introduced by rotations of the heads about their long axes, a motion that would not be easily detected by electron microscopy of X-ray diffraction. This possibility has been explored quantitatively and it has been shown that isotropic orientations of probes can be achieved by allowing free rotation of the myosin head within a cone whose full angular spread is about 40°.[252] This, however, requires rather specific orientation of the probe axis relative to the long axis of the head. A third explanation is that considerable cross-bridge disorder is also predicted by the diffraction patterns.

X-ray diffraction patterns from relaxed and rigor frog muscle showed diffuse scattering at low angles that was attributed to disordered cross-bridges.[215] The radial intensity distribution of this scattering in relaxed fibers resembled that found for pure S-1 in solution. Stretching a relaxed muscle resulted in a decrease in the intensity of the myosin based layer lines and an increase in the intensity of the diffuse scattering. When stretched fibers were put into rigor the myosin layer lines completely disappeared and the diffuse scattering increased further. From these changes one can calculate that in relaxed muscle at rest length the diffuse scattering accounts for approximately 30 to 50% of the myosin heads. This quantitation is only approximate because soluble proteins will also contribute to the diffuse scattering and their exact contribution is difficult to estimate. The fraction of well-ordered cross-bridges can also be estimated by measuring the absolute intensity of the myosin layer lines relative to the undiffracted beam. In one preliminary report of such a measurement the intensity of the 14.3 nm meridional reflection was determined to be only 0.6% of the intensity expected if all of the cross-bridges were well aligned at intervals of 14.3 nm along the thick filament in relaxed muscle.[253] If this result is substantiated by a more complete study then there must be considerable disorder in relaxed cross-bridges to account for the low intensity of the 14.3 nm meridional reflection.

No simple picture of cross-bridge configurations arises out of the data described above. This may be due to inadequacies and artifacts associated with the methods used, or it may be indicative of complexities inherent in the configurations of the cross-bridges. Assuming that the latter explanation is correct it is possible to design a hypo-

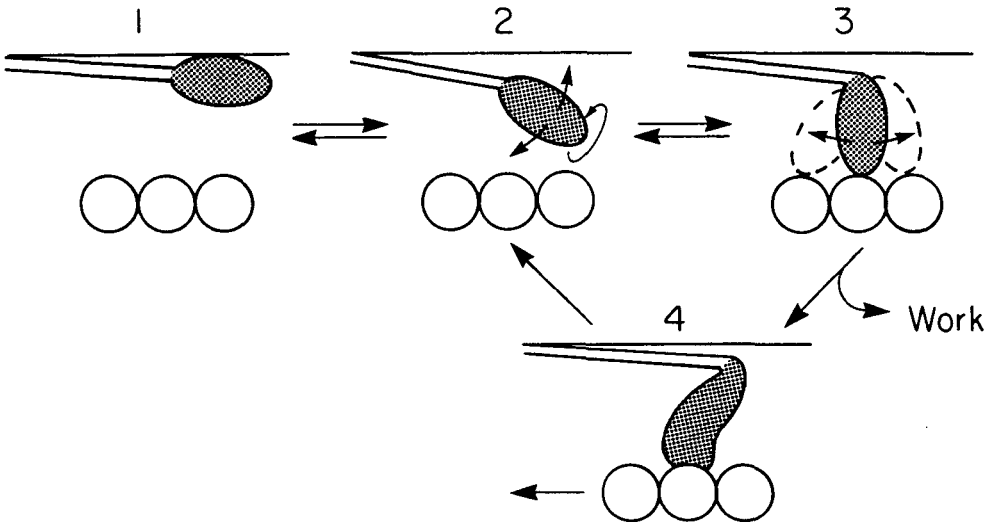

FIGURE 8. Cross-bridge configurations for which some evidence is presented in the text. Configurations 1 through 3 are present in relaxed fibers. In configuration 1 the myosin heads are bound to the thick filament backbone in an ordered array as determined from the intensities of the myosin based layer lines observed in the small angle X-ray diffraction patterns. Configuration 2 depicts a myosin head that is not attached to either the thin or thick filaments and is executing Brownian rotations about all three axes with correlation times of 1 to 10 μsec. The cross-bridge can also attach transiently to actin and may have the freedom to attach to several actin monomers as shown in configuration 3. In the text, configurations 1, 2, and 3 are termed the ordered configuration, the disordered configuration and the attached configuration, respectively. All three configurations are presumed to be in rapid equilibrium in relaxed fibers. A configuration in which the cross-bridge is attached weakly to actin in a rather disordered fashion is thought to represent the beginning of the powerstroke and it is possible that attached cross-bridges in relaxed fibers at low ionic strength resemble this configuration. Although these configurations are probably not exactly the same, their differences have not been defined and they are here depicted together in configuration 3. There is good evidence that in the absence of nucleotides the myosin head attaches firmly to actin in an angled configuration, as shown in configuration 4. This configuration is most probably found at the end of the powerstroke. Thus work would be performed in a contracting muscle by the transition from the weakly attached configuration 3 to the strongly attached configuration 4. Configuration 4 would then be detached to configuration 2 which would reattach in configuration 3 in a subsequent cycle. In the schematic shown above, the powerstroke is depicted, hypothetically, as a conformational change within the myosin head, which is shown in more detail in Figure 9.

thetical model that is consistent with all of the data. Different experimental results have suggested three different cross-bridge configurations in relaxed muscle, and a simple assumption is that these coexist, shown schematically in the upper row in Figure 8. In one configuration they are aligned along the thick filament (the ordered configuration, number 1 in Figure 8); in one state they are transiently and weakly attached to actin (the attached configuration, number 3 in Figure 8); and in one they are executing Brownian rotations (the disordered configuration, number 2 in Figure 8). Transitions among these structural states are probably very rapid. In the ordered state the heads are positioned so that they do not interact directly with actin. Because Ca^{++} has no effect on this ordered configuration it is difficult to understand how cross-bridges receive a signal to commence interaction with the thin filaments.[13,220] The demonstration that, in relaxed muscle at low ionic strength, myosin can bind to thin filaments explains how activation is sensed; heads bind transiently and weakly and then continue to progress through the other states in the contractile cycle unless prevented by tropomyosin. The presence of an attached structural state is supported by electron micrographs showing attached heads and by the high stiffness of relaxed fibers at low ionic

strength. The passage of heads between the ordered and attached configurations requires an intermediate disordered structural state where the flexibility of the myosin molecule allows it to find the binding sites on the thick or thin filaments.[55] The presence of this configuration is shown by diffuse scattering of X-rays and by the angular disorder seen by probes.

This model can also explain a number of observations of cross-bridge order and motion in isolated thick filaments. Both the ordered and disordered configurations of cross-bridges are found in electron micrographs of isolated thick filaments depending on the mode of preparation. If the filaments are first allowed to settle onto hydrophilic grids and then stained, disordered heads extend from the filament backbone as shown in Figure 2.[55] If the filaments are stained over holes or on hydrophobic grids the ordered state is more readily seen, as shown in Figure 6.[239,240,242] The preparative procedures for electron microscopy could favor the ordered configuration (hydrophobic grids) or the disordered configuration (stain after attachment to a hydrophilic grid). The decay of phosphorescence anisotropy is biphasic, and the component with rapid rotational times could be associated with the disordered state while the component with slower rotational times or the component that does not appear to rotate at all would be associated with the ordered configurations.[135]

C. Intermediate States in the Cross-Bridge Cycle

As currently envisioned, force is produced in a complex cycle of states that involves alternate binding and dissociation of a myosin head to and from actin. The head binds to actin in one specific orientation at the beginning of the powerstroke. It changes its conformation, pulling the actin into the center of the sarcomere by some 5 to 10 nm, finally reaching the end of the powerstroke in a second different and specific orientation. It is then released, spends some time in detached states, and rebinds to actin to commence another cycle. Much effort has been expended in attempts to identify and study the different steps in this cycle.

A number of workers have hypothetized that the orientation of the myosin head depends on the properties of the nucleotide bound to myosin, and this has been pursued by measuring cross-bridge orientations in the presence of nucleotide analogs. An obvious addition is ADP, which might produce states that preceed the end of the powerstroke. Addition of ADP to rigor muscle caused a small, less than 1 nm/half sarcomere, increase in the apparent length of the muscle, with no change in stiffness.[254] The length change could be indicative of a change in cross-bridge configuration, however, the magnitude of this change is small and its relation to cross-bridge configuration is difficult to estimate. An apparent lengthening of the muscle could also result from a redistribution of attached bridges. The stiffness measurements have been taken to indicate that no cross-bridges were dissociated by ADP. Addition of ADP caused no change in the X-ray diffraction pattern.[255] The orientations of paramagnetic probes and of several fluorescent probes all attached to SH_1 do not change upon addition of ADP nor does the orientation of muscle tryptophans.[217,256,233] In contrast, the orientations of two fluorescent probes attached to SH_1 do change upon addition of ADP.[245] It is strange that different probes, all attached to SH_1 report such diverse results. It is possible that some probes are insensitive to an ADP-induced change in the conformation of the head because their principle axis is oriented perpendicular to the plane of rotation. However, the paramagnetic probes are sensitive to rotation about any of their axes because the interaction between a nitroxide spin probe and the magnetic field is described by a tensor that is approximately but not exactly cylindrically symmetric. Careful examination of EPR spectra has shown that no probe axis has been rotated by more than 10° by addition of ADP.[256] Alternatively, ADP binding may cause one domain of myosin to shift orientation while a second domain remains rigidly oriented

on actin; this movement may be sensed by some probes but not others. Numerous results cited in Section IV, including the distance measurements between SH_1 and SH_2, and the mobility of paramagnetic probes attached to SH_1, show that nucleotides do in fact cause changes in the protein structure near SH_1. Thus a likely interpretation of the probe data is that SH_1 lies at the interface between two protein domains, one of which is tightly bound to actin and is insensitive to ADP binding, while the orientation of the second is changed by ADP. Another method also found that ADP affects the structure of the myosin cross-bridge. Binding of ADP to myofibrils increased the suspectibility to proteolytic digestion of the hinge region of myosin, as well as regions within the head.[257,258] In summary, there is good evidence that some alteration in cross-bridge structure occurs when ADP binds. The lack of changes in X-ray diffraction, stiffness, and in the orientation of some probes argues that ADP does not produce a large reorientation of the myosin head. The state with bound ADP is probably best approximated by configuration 4 in Figure 8.

One nucleotide analog, AMPPNP, in which the oxygen bridge between the terminal phosphoryl groups is replaced by nitrogen, has been the object of numerous studies. The structure of this nonhydrolyzable analog closely resembles that of ATP.[259] When AMPPNP bound to the nucleotide site of myosin in rigor fibers it caused slight elongation of the fiber (2 nm/half sarcomere) and no change in the stiffness.[254,260] As with ADP, the stiffness data were taken as an indication that no myosin heads have detached from actin. However, stiffness may not be a linear function of the number of myosin heads attached to actin if some attached heads are less stiff than others. If the elastic element resides in some portion of S-2, myosin attached by a single head may be as stiff as doubly attached. The X-ray diffraction patterns of fibers in the presence of AMPPNP at 4°C showed both thin filament and thick filament based reflections.[261] For instance, the 14.5 nm meridional reflection, characteristic of relaxed muscle, was strong while a strong 38 nm actin based layer line indicated that myosin heads were still attached to actin. The pattern of reflections could not be obtained by a simple addition of the relaxed and rigor patterns, showing that the state obtained with AMPPNP was a new state not a mixture of rigor and relaxed bridges. Electron micrographs of thin sections of insect flight muscle in the presence of AMPPNP did not show the obliquely oriented cross-bridges characteristic of rigor ribers, although diffuse chevrons and some polarity to the bond remained.[209,210] More bridges were seen attached to actin than in relaxed muscle, and the bridges were not distributed along the actin in the highly ordered pattern of either rigor or relaxed muscle. This pattern is thus in qualitative agreement with the interpretation of the X-ray diffraction data in that states intermediate between rigor and relaxation are seen.

Spectra of paramagnetic probes attached to SH_1 show that in the presence of AMPPNP, a fraction of the heads remain bound to actin with probes oriented as in rigor, and the remaining heads appear to be isotropically distributed as in relaxed muscle.[219] Saturation transfer EPR spectra confirm that there is a fraction of probes with mobility in the 1 to 10 μsec time range which corresponds to the disordered fraction and strongly suggests that this fraction represents heads that have been detached from actin by the binding of AMPPNP.[262] As described previously, the binding of actin protects the region between the 50 kD and 20 kD fragments from digestion. Digestion of skeletal and cardiac myofibrils in the presence of AMPPNP showed two digestion patterns, one characteristic of attached heads of rigor fibers and one characteristic of detached heads of relaxed fibers.[258,263]

No single clear picture of cross-bridge configurations in the presence of AMPPNP emerges from the above data. The general consensus from electron micrographs, X-ray diffraction patterns, and measurements of stiffness is that heads remain attached to actin in the presence of AMPPNP, but that they have different preferred orienta-

tions and axial distributions. This interpretation is weakened if some fraction of heads are detached by AMPPNP, as suggested by both the probes and the digestion patterns. It is probable that in this case there would be many myosins with only one head attached, and the effect of single-headed attachment on fiber stiffness, X-ray diffraction, or the electron micrographs is not known. Alternatively, the probes on SH_1 or at the nucleotide site may not be sensitive to large changes occurring in other portions of the head. The demonstration that tension increases the affinity of the myosin head for AMPPNP is evidence that AMPPNP causes a structural change in myosin heads that are attached to actin.[260] It is not likely that this transition involves a rotation from 45 to 90° as was originally thought.[8]

States similar to those of relaxation can be produced by a variety of nucleotide analogs. As shown by X-ray diffraction and mechanical measurements, relaxation was induced by two ATP analogs, one in which a sulfur replaces an oxygen on the terminal phosphoryl (ATPγS), and one in which a CH_2 group replaces the oxygen bridging the α and β phosphoryls.[261] Both analogs are hydrolyzed slowly by myosin. A relaxed state was also produced by addition of AMPPNP in the presence of 50% ethylene glycol.[264] ADP and vanadate form a tight complex, thought to mimic the $ADP \cdot P_i$ complex, and they also transform the tension, stiffness, and the X-ray diffraction properties to those characteristic of relaxed muscle.[265] The observation that all these analogs produce X-ray diffraction patterns similar to those from relaxed muscle shows that, at least within the resolution of this technique, cleavage of ATP does not alter the conformation of the detached head.

At low ionic strength in the absence of Ca^{++} cross-bridges appear to attach to actin without generating force.[235,236] Such a weakly attached cross-bridge state possibly represents a configuration at the beginning of the powerstroke, as shown in Figure 8. X-ray diffraction patterns obtained from fibers relaxed in low ionic strength were different from either relaxed or rigor fibers.[266,267] Many myosin based reflections remained, showing that some cross-bridges were ordered on the thick filament, while some additional intensity was seen on the 5.9 nm actin based layer line. This intensity was further from the meridian than in rigor suggesting that the attached cross-bridges were more disordered than those attached in rigor. In a preliminary report, paramagnetic probes on the SH_1 also showed an ordered fraction in low ionic strength relaxing solutions; however, the probes in this fraction were more disordered than those on rigor bridges, again suggesting a weak disordered bond between myosin and actin.[268] The mean angle of these probes in the ordered fractions was similar in rigor and low ionic strength relaxation so that the probes gave no indication of a large change in orientation between these states. This observation is consistent with a model in which the orientation of the region of myosin in the vicinity of SH_1 is largely determined by binding to actin.

The data obtained in relaxed fibers at low ionic strength indicates that myosin attaches weakly to actin in a configuration that is clearly different from the rigor one. Weak attachment to actin may also be occurring at a higher ionic strength in the presence of ADP and vanadate. The complex of these ligands with myosin is thought to be an analog of the state which first attaches to actin in the contractile cycle. Although the X-ray diffraction pattern resembled that of relaxation, some interaction with actin was indicated by the observation that the $ADP \cdot V_i$ complex was released from myosin much faster in the fibers than in solution.[265] Studies performed in solution with actin and myosin subfragments have suggested that when myosin is in a complex with ATP or $ADP \cdot P_i$ it attaches weakly to actin (discussed in Section VI). The constraints imposed by the filament lattice may make the population of attached states in the presence of these nucleotides even lower in the fibers than in solution.

In summary, there is reasonable evidence that cross-bridges can attach weakly to actin in relaxed muscle at low ionic strength and also in the presence of ADP and

vanadate. These states are depicted by configuration 3 in Figure 8. Although the orientation of these cross-bridges is not well characterized, it is different from the rigor configuration (configuration 4 in Figure 8). The binding of AMPPNP may produce a change in the orientation of attached bridges, however, there remain a number of ambiguities in this interpretation. The binding of ADP most probably causes very little change in cross-bridge orientation.

D. Cross-Bridge Configurations in Active Fibers

Activation of skeletal muscle produces a dramatic change in the muscle cross-bridges. Force, ATPase activity, and stiffness all increase by about three orders of magnitude. Activation also results in large changes in the X-ray diffraction pattern which show that myosin cross-bridges have moved away from the thick filaments and closer to the thin filaments and that they have lost much of the order found in either resting or rigor muscle.[13,269,270]

Upon activation the intensities of the equatorial reflections changed from their relaxed values towards their values in rigor indicating that a large fraction of the cross-bridges moved from the vicinity of the thick filament to the vicinity of the thin filament.[269] The intensities of these reflections are determined by the distribution of mass in the equatorial plane. Recently, a consideration of the possible phases of the first five equatorial reflections has produced a picture of the redistribution of mass with higher resolution than could be obtained with only two equatorials.[271] The mass transfer involved both radial and azimuthal components and the net transfer was equivalent to 20 to 40% of the total mass of the myosin heads. This could be an underestimate of the fraction of cross-bridges transfered from the vicinity of the thick filaments to that of the thin filaments.

Activation also induced a large decrease in the intensities of the myosin based layer lines. The intensity of the first layer line fell to about 20% of its resting value, and similar, but not equal, changes occurred in the intensities of the other layer lines.[13,269,272,273] The intensity of a myosin based layer line is proportional to the square of the population of myosin heads giving rise to it so that these results show that about 50% of the heads that were associated with the helical array of the thick filament have now left this array. The binding of myosin heads to actin should produce additional intensity on the actin layer lines, however, for a number of years no such enhancement could be detected.[13,269] The failure to detect the enhancement of the actin layer lines was interpreted as an indication that the attached myosin heads were disordered, but an alternative explanation, that the fraction of attached heads was small (<20%), could not be ruled out. Recently, two investigations using improved techniques, have found that the intensity of the 5.9 nm actin layer line was enhanced during activity.[274,275] This important result shows that a relatively large fraction of myosin heads have attached to the thin filament. The distribution of additional intensity along the layer line had not shifted towards the meridian as occurs in rigor fibers. Thus, the additional mass which is enhancing the actin reflections is close to the actin filament. The failure of the attached myosin heads to enhance the other actin reflections could be explained if they attach to actin in a variety of orientations such that disorder in the regions distal from actin greatly weakens the higher order scattering.

Although the myosin based layer lines all decreased in intensity during isometric force generation, one reflection, the 14.3 nm meridional, exhibited a more complex behavior.[273] The integrated intensity of this reflection first decreased, then increased to a value that was about 50% greater than at rest, and finally decreased again during relaxation. The increase in intensity is of particular significance. It shows that the distribution of cross-bridge mass is aligned axially with a stronger 14.3 nm periodicity than in resting muscle. Although the population of the helical array of ordered cross-

cleotide in contracting fibers was the same as that found in rigor fibers in the presence of the diphosphate analog. These data suggest that the orientation of the nucleotide site also does not change during the powerstroke, a conclusion similar to that drawn from paramagnetic probes attached to SH_1.

It is highly unlikely that at any instant all attached myosin heads could be at the end of their powerstrokes in active isometric muscles, as deduced from the probe spectra discussed above. Rather, it is more likely, as discussed later, that the attached heads are distributed among the various states within their powerstrokes. Thus, the above results, which find only one probe orientation on heads that are attached to actin, suggest that the probe orientation does not change during the powerstroke. There are two trivial explanation of these results. Neither a pure azimuthal rotation, nor a rotation that is symmetric about 90°, will be detected by any of the probes due to the symmetries of the fiber. However, neither explanation appears likely. Azimuthal rotations would not be an efficient mechanism for generating axial forces. The possibility that force is produced by an axial rotation that is symmetric about 90°, i.e., from 90 $-\theta$ to 90$+\theta$, is unlikely because different probes that have various inclinations to the fiber axis give the same results. Alternative explanations of this data would have to postulate that there are myosin heads attached to actin with probe motions and orientations that are indistinguishable from the disordered and mobile heads observed for heads that are not attached to actin. Although weakly bound states, such as shown by configuration 3 in Figure 8, may possess these characteristics, heads in active powerstrokes must be strongly bound to actin and would not be expected to exhibit such disorder and motion.

Data discussed previously also supports the conclusion that a portion of the myosin head, the portion containing the SH_1 and the nucleotide, is not involved in the conformational changes that occur in the powerstroke. Probes at these sites were well-ordered in rigor muscle, yet the myosin molecules must undergo considerable distortion in order for them all to attach to actin. The paramagnetic probes also did not sense any changes in orientation that may be induced by AMPPNP or ADP. As discussed in Section IV, the distance between SH_1 and Cys-374 on actin was unchanged by addition of AMPPNP or ADP.[171] The most simple interpretation of all of these data is that there is a portion of myosin whose orientation is fixed by its attachment to actin. The size of this oriented portion is rather large because neither the myosin nucleotide binding site nor SH_1 are close to actin,[170-172] and direct visualization of a biotin labeled SH_1 shows that it is approximately 7 nm from the tip of the myosin head.[282]

The fraction of myosin heads that are attached to actin during isometric force generation has been estimated by several techniques, which have given a broad range (20 to 70%) for vertebrate muscle. The magnitude of the attached fraction has been given much attention because it influences the interpretation of many results, including X-ray diffraction patterns, probe spectra, and energetics, and it is a powerful constraint for models of cross-bridge kinetics. Most measurements involve a comparison between rigor, relaxed, and active muscle with the assumption that the properties of attached cross-bridges are similar in rigor and activity, an assumption that is probably not valid and may explain some of the disparity in results. The stiffness of single frog fibers during contraction was 70% of the stiffness found in rigor.[283] Stiffness often correlates with tension and has been commonly assumed to be proportional to the fraction of attached bridges, but this assumption may not be valid when comparing rigor and active muscle. The change in the ratio of the equatorial intensities accompanying the transition between the relaxed and active states was about 50% of that which occurs during the relaxed to rigor transition.[13,269] An attached fraction of 50% was also estimated from the dichroisim of probes attached to SH_1; however, since these spectra only measure a single orientation dependent parameter it is difficult to decompose

them into two components.[245,246] As discussed above, EPR spectra estimate that only 20% of cross-bridges are attached during isometric contraction.[262,280] Unlike the other estimates, in which a single parameter is assumed to behave linearly between the resting and rigor states, EPR spectra resolve two fractions, and uncertainties in the estimate arise from the identification of the disordered component with detached heads. An entirely different type of estimate comes from measurements of muscle energetics. During isotonic contractions the time that a head is attached in an active ATP splitting powerstroke is proportional to the length of the powerstroke divided by the velocity of contraction. The fraction of heads attached is given by the time attached in one powerstroke divided by the time required to complete one cycle. If the heads operate independently in their powerstrokes then the above calculations estimate the fraction of attached heads. When measurements of the ATPase activity of isotonically contracting live frog fibers are inserted into this expression, one obtains a value of 14% attached in powerstrokes at about 0.3 lengths per second which, when extrapolated to isometric contractions, gives an estimate of about 25 to 30% attached and generating force.[262,284] The error in this estimate arises mainly from the uncertainity in the length of the powerstroke. Attachment of more than 50% of the heads would require a powerstroke of more than 20 nm, which is unlikely. Some of the disparity in measurements made with different techniques may be due to the particular fiber preparations used. The tension generated by glycerinated and labeled rabbit psoas fibers, approximately 0.2N/mm^2, is lower than the 0.3 N/mm^2 generated by living frog muscle.[23] The estimates of the attached fraction provided by probes may therefore be lower by a factor proportional to the ratio of tensions. However, some of the disparity could also rise if a fraction of the heads are attached weakly in states not involving ATP hydrolysis.[285] Such heads could contribute to changes in equatorial reflections or stiffness and could enhance the intensity of the actin based 5.9 nm layer line but they may not be sensed by the EPR probes or by measurements of energetics. From the estimates obtained by paramagnetic probes and energetics, it appears most likely that in active living vertebrate skeletal muscle 25 to 50% of the myosin heads are tightly attached to actin in active powerstrokes. There is probably an additional fraction of heads that is attached more weakly to actin, with greater disorder than the strongly attached heads.

The interpretation of a number of results also strongly depends on the distribution of cross-bridges within the powerstroke during isometric contractions. This distribution is usually described in terms of a variable x, which denotes the axial distance between a cross-bridge and a site for attachment to the thin filament. Several results argue that the ensemble of cross-bridges is distributed evenly throughout the states of the powerstroke during isometric contractions. This can be seen most easily by a comparison of isometric contractions and slow isotonic contractions. When fibers contract at velocities such that the time to traverse the powerstroke is short compared to the inverse of the isometric cycling rate, the probability for attachment or detachment during the powerstroke is small and thus the majority of cross-bridges traverse the entire powerstroke, giving a rather uniform distribution of bridges within the powerstroke. For a cycle rate of 1 sec^{-1} and a powerstroke of 10 nm a uniform distribution is obtained at velocities greater than 0.05 to 0.1 muscle lengths per second. Put another way, if cross-bridges are distributed unevenly in attached states in isometric fibers, and cross-bridges are cycling in and out of this distribution at a frequency of 1 sec^{-1}, then this distribution will be radically altered when the fiber moves at a velocity of 0.05 to 0.1 muscle lengths per second. Thus the comparison between isometric fibers and those executing very slow isotonic contractions provides an important insight into the cross-bridge distribution. The experimental observation is that most parameters of cross-bridge configurations do not change between these two cases. In such slow contractions the force, stiffness, and ATPase activity are almost the same as found in isometric

contractions.[284,286,289] In particular, if the force produced by a cross-bridge varies with x, which is rather likely, and the distribution also varies with x, then the transition from isometric to slow isotonic contractions should result in a large change in force. The transient tension response to step changes in length was not greatly different in isometric and slow isotonic contractions although the response was considerably faster at higher velocities.[288] This response should be very sensitive to cross-bridge distributions.[288] In X-ray diffraction patterns both the ratio of the equatorial intensities and the intensity of the 14.3 meridional reflection are unchanged in slow isotonic contractions.[272,289] However, one study has found a decreased intensity of the 14.3 reflection during constant velocity contractions.[290] Taken together the data show that cross-bridge configurations are not significantly changed by slow movements, leading to the conclusion that cross-bridges are distributed evenly throughout the force, producing states of their powerstroke during isometric contractions.

An even distribution of cross-bridges throughout their attached states would be expected if the filaments were not exactly stationary with respect to each other during isometric contractions. If fluctuations in the axial alignment of thick and thin filaments exceed 10 nm in times less than 1 sec they will even out any tendency for cross-bridges to pile up at one point in the powerstroke. It is very unlikely that filaments remain in precise axial alignment during isometric contraction. The number of force-producing cross-bridges on either end of a thick filament may vary stochastically, producing fluctuations in axial tensions and in the position of the filament. Fluctuations in the position of structural subunits of the sarcomere are detected during isometric force generation by quasi-elastic light scattering,[291] and fluctuations in tension have been measured directly.[292] A reasonable model of the isometric state is one in which small rapid fluctuations in the relative positions of the filaments smear out the distributions of the attached cross-bridges and also lead to cross-bridge cycling and the hydrolysis of ATP. This model would explain why faster muscles, which would have more rapidly fluctuating filaments, have a greater isometric ATPase activity. Alternate models explain the isometric cycling rate in terms of attachment and detachment of cross-bridges within the powerstroke with little fluctuations in filament position.[293] In support of this view, a preliminary study has found that the tension of individual myofibrils does not fluctuate, and further work is needed to differentiate between the two above models.[294]

The results discussed above have all been obtained during steady isometric or isotonic contractions, and considerably more information can be obtained when cross-bridge distributions are perturbed by step changes in muscle length. When active fibers are shortened rapidly by 1 to 10 nm per half sarcomere, rapid and complex changes occur in muscle tension.[295] Thus a rapid transient in muscle length allows one to perturb the cross-bridge distribution, to then observe it as it relaxes back to the steady state, and thus obtain information on the rates of mechanical transitions occurring within the powerstroke. An almost instantaneous decrease in tension has been attributed to elastic elements that have been shown to reside primarily within the cross-bridge.[296,297] If the decrease in muscle length was small, less than 4 nm per half sarcomere, the tension returned rapidly to its original level within a few milliseconds. The fast tension recovery was attributed to a rapid transition between states of attached cross-bridges within the powerstroke. This assumption was supported by the observation that muscle stiffness does not change during the first rapid recovery of tension, showing that the net number of attached cross-bridges have remained constant during the recovery of force.[298] The initial drop and rapid recovery of tension could be explained quite well by a model in which a cross-bridge could attach to actin in several configurations, with transitions between configurations stretching an elastic element.[295] Following a step decrease in length the tension drops due to shortening of the

elastic elements. The rapid recovery phase is then due to transitions between the two states that reextend the elastic element. The model requires a nonuniform distribution of cross-bridges within the states, with the population of states early in the powerstroke being higher, and as discussed earlier, such a distribution is unlikely.

Another type of transient experiment has been performed in which the load applied to the muscle was abruptly changed, and the transients in the length of the muscle were observed.[299] These experiments led to a model in which rapid association of cross-bridges occurred at the beginning of the powerstroke and rapid dissociation of cross-bridges occurred at the end of the powerstroke.[300] Recent experiments have supported this model. The response of a fiber to sinusoidal oscillation can be resolved into three rates, one of which is thought to correspond to the rapid recovery of tension following a step decrease in length, and this rate constant was slower at lower concentrations of ATP.[301] In agreement with this conclusion, direct measurements using insect flight muscle show that the latter portion of the force recovery following step changes in length is slower at lower concentrations of ATP.[302] Both of these results can be interpreted as an indication that rapid dissociation and association of cross-bridges occurs during this phase. Alternatively, the data at low ATP could be explained if the binding of ATP led to a state that produced a large force.[302] However, there is no evidence for a force-producing actomyosin·ATP state, and evidence presented in Section VI argues against such a model. A rapid dissociation of cross-bridges following step changes in length is expected if cross-bridges are distributed throughout force-producing states before the change in length, as argued above. In this case, the length change would necessarily carry some attached cross-bridges into states beyond the powerstroke, and these states probably produce a negative force and are rapidly detached by ATP. Data discussed in Secton VI show that myosin heads are in rapid equilibrium between attached and detached states with transitions that are sufficiently fast that they could play a role in the transient recovery of tension. Thus the transients which follow step changes in either length or load are probably a product of both events that occur within the powerstroke and the rapid attachment and detachment of cross-bridges at either end of the powerstroke. If this is the case, the interpretation of these transient experiments is rather difficult due to the inability to resolve these two processes.

When a muscle is shortened so that the relative filament movement is 4 to 12 nm the rapid recovery of tension is not complete and is followed in some cases by a further decrease in tension before returning to the original value.[295 297] For step changes in excess of 12 nm no rapid recovery of tension occurs. This observation, coupled with the observation that the sarcomere can shorten very rapidly for 12 nm before commencing steady shortening, has been taken as an indication that the powerstroke is 10 to 12 nm in length.[303] This assessment of the length of the powerstroke is model dependent, however. It would be accurate if during isometric contractions most cross-bridges were found at the beginning of their powerstrokes, and simply executed the powerstroke following the change in length. In the event that they are distributed throughout the force-producing states, and some are dissociated during the transient, the actual length of the powerstroke is not related in a simple fashion to the above data.

Although the exact nature of the events that follow step changes in fiber length remain undetermined, this experimental protocol nonetheless allows one to perturb the cross-bridge distribution and to observe it as it relaxes back to the steady state. Recent experiments have involved observations of X-ray diffraction patterns using synchrotron radiation. Following a quick release of 10 nm per half sarcomere, the intensity of the 14.3 nm meridional reflection decreased dramatically to 10 to 20% of its isometric value.[13,272,304] The recovery of intensity was biphasic with a fast phase, comprising two thirds of the total, that was complete in 6 msec, followed by a slower return to the initial value. The recovery of tension followed a slow time course similar to that of the

slow recovery in intensity. Thus, little change in tension occurred in the first 6 msec when the changes in the intensity of the meridional reflection were more pronounced. Rapid restretch of a shortened fiber caused a rapid recovery in intensity during the first 1 to 2 msec but caused a subsequent decrease in intensity if applied later in the recovery. A similar decrease in intensity followed a quick stretch, however, the increase in intensity and return of tension occurred in parallel. These data show that sudden length changes cause a loss in the periodic alignment of cross-bridge mass along the 14.3 nm repeat of the thick filament. The strength of this periodicity has been increased by activation as discussed previously, and attributed to tension generated alignment of portions of the myosin head on the cross-bridge repeat. The interpretation of these results is difficult because it is not known which portion of the cross-bridge is aligned on the 14.3 nm repeat, nor how cross-bridge distributions are changing during the first 6 msec of the recovery. If the intensity of the 14.3 nm reflection is due to alignment of the region of myosin at the junction of S-1 and S-2, then rapid changes in length create axial disorder in this region as might be expected if cross-bridge orientation does change in the powerstroke. However, one result indicates that extensive rotation of a large fraction of cross-bridges does not occur during the transients in tension. The ratio of the equatorial intensities remains constant, within 10%, during the transient, and rotation towards a more acute angle would be expected to bring the cross-bridge mass closer to the thin filament and thus enhance reflections that originate from planes that contain the actin filament.[13,272] The orientation of fluorescent nucleotides does not change following the rapid changes in muscle length,[305] again showing that probes in this region of myosin do not sense alterations in protein orientation during the powerstroke. The change in the birefringence of the fiber following step changes in length is less than 2% of the value obtained in resting muscle, suggesting that a large reorientation of protein structures has not occurred.[306]

E. Models of the Powerstroke

The powerstroke is defined as the set of transitions of the nucleotide-actomyosin complex in which chemical energy is transformed into work. This transformation occurs during the relative movement of the filament through a distance of order 10 nm, as force is applied by the myosin head. The transitions that drive this process must involve large changes in both the potential energy associated with the protein structures and in the structures themselves. Models for these changes are discussed briefly below in light of the results outlined in the previous sections, and the interested reader should refer to these sections for more complete descriptions and citations.

The first models of the powerstroke, derived from electron micrographs and from X-ray diffraction invoked the rotation of the entire myosin head. More recently the probe data have indicated that a portion of myosin, adjacent to actin, does not rotate during the powerstroke. A simple addition to the earlier model, which will account for the data from probes, is to confine rotation to some portion of myosin distal from actin while a portion proximal to actin, which contains the probes, remains fixed. Is there any evidence that some portion of the myosin head rotates during the powerstroke? More recent electron micrographs of rigor muscle show some variation in the conformation of attached cross-bridges, suggesting that distortion has occurred in order to form the rigor bond. If the variations in orientations are not artifacts of the preparation, and monitoring the preparation using X-ray diffraction suggests that they are not, they provide clear evidence for distortion of the orientation of a region of myosin distal from actin. Recent evidence obtained from X-ray diffraction patterns of active muscle also suggest that the myosin heads attach to actin in a disordered fashion. The enhancement of the 5.9 nm actin layer line without enhancement of the other layer

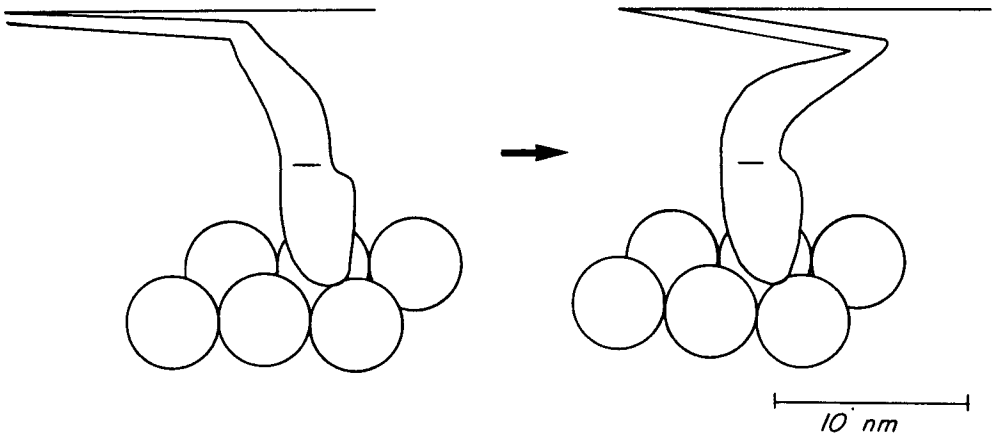

FIGURE 9. A hypothetical model for the changes in cross-bridge conformation that occur during the powerstroke. The configuration shown on the left represents the beginning of the powerstroke. The cross-bridge shape at the end of the powerstroke, depicted on the right, is traced from the reconstructions of electron micrographs of S-1 crystals shown in Figure 3. It is assumed that the rather massive end of S-1 attaches to one or more actins and has an orientation that remains constant during the powerstroke, while the orientation of the narrow neck region changes. Although the figure depicts a change in the shape of this neck region, it should be noted that a rotation of this region about a vertical axis in the figure would move its distal end by a sufficient distance to account for the powerstroke. As described in the text, there is considerable evidence that suggests a change in orientation of a portion of the cross-bridge distal to actin while a proximal portion remains unchanged. The assignment of these portions to distinctive regions of the structure of S-1, however, is speculative.

lines shows that myosin heads have bound to actin but that they do not have the orientation found in rigor. Because the enhancement of the layer lines during activity occurs further from the meridian than in rigor, the ordered portion of the myosin head must be close to actin and the portions distal from actin must be disordered. Thus, a synthesis of the more recent results of electron microscopy and X-ray diffraction suggests that a portion of myosin distal to actin changes orientation during the powerstroke.[307] Several results obtained in solution also support this model. Fluorescence energy transfer within the actoS-1 complex finds that SH_1 does not change its distance from actin in response to the binding of nucleotides, while a second site on a more distal portion of S-1 does change its distance. Electron micrographs of cross-linked actoS-1 in the presence of ATP suggest changes in orientation of at least a portion of S-1. In summary, there appears to be reasonable evidence that rotation of some portion of myosin, distal from its attachment to actin, does occur during the powerstroke, as shown in Figure 9. Such a model is also consistent with a number of other pieces of evidence. Force would be generated by a change in the conformation of some protein structure near the middle of the myosin head, and this is where the nucleotide binds and where a considerable movement of two SH groups has been observed. A change in the orientation of the less substantial neck region of the head may not produce a large alteration in the X-ray diffraction pattern, explaining the lack of change seen in the equatorials during the transient response to length changes in active fibers and to application of stress in rigor fibers.

The main argument against a model in which one portion of myosin changes orientation while a second is stationary is the lack of evidence that the myosin head changes its shape in response to the binding of nucleotides or actin. In particular, neither actin nor nucleotides caused a change in the distance between SH_1 on the stationary portion of myosin, and cys-177 of the A-light chain on the distal region of myosin which is

supposedly the region that changes its orientation. However, methods used to obtain this and other negative results may not have been sensitive to changes that did occur, so that these results do not preclude this model. Recently, an analysis of the electric birefringence of S-1 has led to the conclusion that S-1 is composed of at least two domains that can move relative to one another in response to strong electric fields.[360] This result supports models such as the one shown in Figure 9.

One question pertinent to the above model, as well as to other models, is whether the conformational changes involve the movement of rather rigid domains relative to one another or whether they involve plastic deformations in large portions of the molecules? For instance, in the model shown in Figure 9 the powerstroke could consist of a simple rotation of the crooked neck region of myosin or in a change in its shape. The results discussed in Section III, which show effects at numerous sites due to binding of nucleotides or upon binding of myosin and actin, argue for at least subtle changes throughout the molecules, in favor of models invoking large-scale plastic deformations. However, at present the data do not provide an answer to this important question and these aspects of the models must remain speculative.

Recently, a new model compatible with the data obtained by a variety of methods, including probes and X-ray diffraction, has been proposed.[285] It is assumed that a large fraction of heads are attached to actin, as indicated by the equatorial reflections, but that only a small fraction of these are involved in powerstrokes. The loosely attached heads are disordered so that they do not contribute to the actin layer lines or to the ordered states seen by probes. A crucial feature of the model is the assumption that the length of the powerstroke is short, approximately 4 nm. As heads enter the powerstroke they rotate to a more acute angle, extending the 4 nm elastic element that resides in the cross-bridge. The powerstroke then involves the shortening of this elastic element, while the myosin head, or at least a portion of it remains rigidly fixed to the actin.

The conformational changes discussed above have been considered in detail because they are simple mechanical models that are supported by a considerable body of the data. Other models, however, have not been excluded by the data. One model, which involves the shortening of S-2, has been discussed in Section IV.C. Although there is no direct evidence that such shortening occurs in active muscle, there is also no evidence that excludes some shortening of S-2, and the inhibition of contraction by antibodies directed against S-2 proximal to the head suggests that this portion of S-2 may be involved in force generation. In another model, contraction involves large changes in the helical array of actin monomers induced by interaction with myosin.[308] Such a model is suggested by mounting evidence that the actin filament also has sites of flexibility that are candidates for active elements.

In conclusion, the data most strongly supports models for the powerstroke in which a portion of the myosin head, approximately the half that is distal from actin, changes its orientation. One model of this type is shown in Figure 9. Although this is an attractive hypothesis, the possibility exists that the actual mechanism may involve changes in conformation that are radically different from any of those contemplated above.

VI. THE INTERACTION OF ACTIN, MYOSIN, AND NUCLEOTIDES

In solution, actin, myosin, and ATP undergo a cyclic interaction involving transient association of the proteins and ATP hydrolysis. This cycle is thought to be similar to that which occurs in a contracting muscle, and it has been studied extensively. At physiological ionic strength both myosin and actin are filamentous and their interaction is constrained by the geometry of the filaments. The soluble myosin subfragments, HMM and S-1, interact with actin in a manner that follows more classic kinetics and

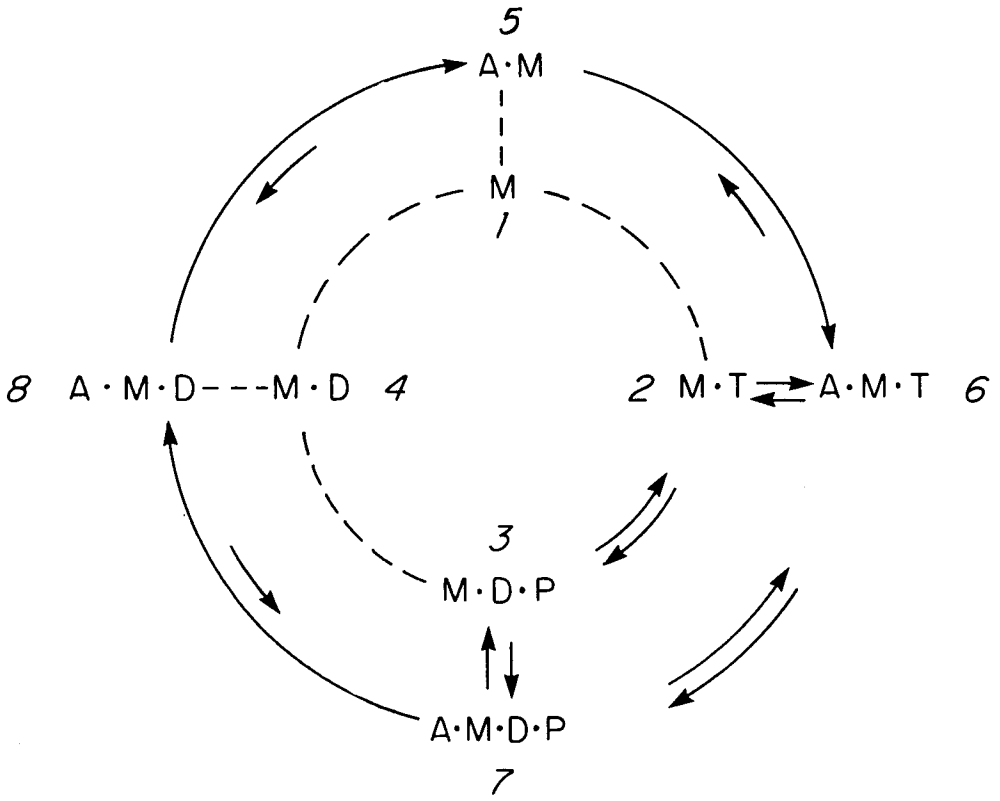

FIGURE 10. Elementary steps in the hydrolysis of ATP by myosin and by actomyosin. Here M represents a myosin head; A, actin; T, ATP; D, ADP; and P, inorganic phosphate. During the steady state cycle of ATP hydrolysis by acto-S-1, the binding of ATP to state 5 is rapid and relatively irreversible. The states 6, 2, 3, and 7 are in rapid equilibrium as indicated by the equal double arrows connecting them. The transitions involving the release of products, or isomerizations leading to the release of products, involve large changes in free energy. In active muscle, work is probably performed by myosin heads attached in states 7 and 8, with states 5 and 6 probably being brief intermediates. The transitions marked by dotted lines occur very rarely during hydrolysis of ATP by actoS-1 or in active muscle. Unequal arrows connecting states indicate that this transition is favored in the direction of the larger arrow.

most studies have used them. A comparison of the interactions of HMM and S-1 leads to the conclusion that the two heads of HMM operate independently. Thus, actin and S-1 form the most elementary system in which to investigate the contractile interaction and this system has been the object of most recent investigations. The kinetics of the interactions of the contractile proteins with nucleotides have been extensively reviewed.[18,22]

A. Interactions in Solution

Myosin alone has a low MgATPase activity which is elevated dramatically by the presence of actin. The overall steps involved, which must include binding of nucleotide, hydrolysis and release of products, are shown in Figure 10. The states shown represent only the elementary steps and each is a composite of many different protein-ligand complexes. Here the word "state" will be used to refer to the complexes shown in Figure 10, while the word "configuration" will refer to the cross-bridge model shown in Figure 8. The first working model of the interactions of actin, S-1, and nucleotides was proposed by Lymn and Taylor.[309] The observation that ATP release of the myosin head from actin was rapid compared to hydrolysis led to a hypothetical

cycle involving the transitions 5→6→2→3→7→8→5 with the release of products 7→8→5 being rate limiting. Subsequent work has modified this proposal as outlined below.

The associations of ATP or ADP with myosin have been shown to take place in several steps.[21,310,311] The first step in the binding of ATP, transition 1→2, involves a collision complex that probably does not produce changes in protein conformation, followed by isomerizations within state two to at least one conformation identified by an increased tryptophane fluorescence.[312,313] The equilibrium constant for this transition is large[310,311] and it occurs with a rate constant that is greater than that of the next step, the hydrolysis reaction. The binding of ADP, transition 1→4, is also followed by isomerizations within state four that resemble those which accompany the binding of ATP.[314] When ATP binds to actomyosin, 5→6, it causes a rapid dissociation of myosin, 6→2, with a rate that exceeds 500 S^{-1}. The dissociation precedes most of the changes in myosin fluorescence or the hydrolysis of ATP[315]. The binding of AMPPNP to acto S-1 produced a change in fluorescence that preceded the dissociation of the proteins.[314] Thus, the binding of nucleotide to myosin is a very rapid multistep process that alters the conformation of myosin so that it has a reduced affinity for actin.

Upon addition of ATP the dissociation of myosin from actin, step 6→2 occurs rapidly, within 3 msec. However, dissociation is not complete, leading to the conclusion that the states 2 and 6 are in rapid equilibrium.[316-318] This initial degree of protein association, measured for skeletal actoS-1 by turbidity, was the same as that at steady state so that the equilibrium between the states 2 and 6 and the states 3 and 7 must be the same. The association of the myosin products complex with actin occurs with a rate constant of 1 to 10 × $10^4 M^{-1}S^{-1}$ in a process that probably involves an initial rapid equilibrium between bound and unbound states followed by an isomerization to a more stable complex.[318]

The hydrolysis step, 2→3, occurs with a rate of about $50s^{-1}$ and with a small equilibrium constant (of order 1 to 10 depending on conditions).[309,319,320] Hydrolysis produces a change in the conformation of myosin that is detected by a further increase in the fluorescence of tryptophane.[321] During the hydrolysis of ATP by myosin alone in the presence of $H_2{}^{18}O$, multiple oxygens are found to exchange into the phosphate released, showing that the hydrolysis step is reversible and that reversal occurs several times before product release.[322] When actin is included the degree of exchange is lower because actin increases the rate of product release via the steps 3→7→8→5.[323] If state 2 binds to state 6 with appreciable affinity and hydrolysis only occurs through the transition 2→3, then the ATPase activity should decrease as the actin concentration approaches infinity. In fact, no decrease was found and a cross-linked complex of actin and S-1 has full activity, leading to the conclusion that hydrolysis by the protein complex, i.e., the transition from 6 to 7 occurs with a similar rate as hydrolysis on myosin alone.[90,316] Thus the hydrolysis step occurs rapidly compared to the overall cycle time, it involves little change in free energy, and it is relatively independent of whether myosin is associated with actin.

There has been considerable discussion concerning the possibility that state 3 is composed of two states. The actin concentration required to produce half maximal saturation of the ATPase activity was several fold smaller than that required for half maximal saturation of the binding of actin and S-1.[317,324] The weak binding of the proteins was explained by introduction of a second state into state 3, so that state 3 consisted of a refractory state, which did not bind to actin and a nonrefractory state which did bind to actin. More recent studies have shown that both of these substrates of state 3 bind to actin so that state 7 would also be composed of two substrates and thus the term refractory no longer refers to an inability to bind to actin but simply identifies a substrate of state 3.[317] It was proposed that the transition between refractory and nonrefractory states would be rate limiting.[325] The primary evidence for these states is the

difference in concentration dependence of ATPase activity and actoS-1 binding, however, extra states have also been postulated to account for the measured rate of ATP dissociation from actoS-1 as well as the rate of oxygen exchange into phosphate.[326,20] The presence of these substrates is still controversial, and some work suggests that the actin dependence of actoS-1 binding and ATPase activity can be explained without them.[327] However, recent studies of the kinetics of cross-linked actoS-1 again suggest that several slow steps must follow the hydrolysis.[328]

During the hydrolysis of ATP by myosin alone the rate-limiting step involves product release and most probably involves a conformational change leading to the rapid release of phosphate.[18,21] ADP is released subsequently in a two step process involving a fluorescent transition in myosin, which has some similarities with the steps occurring upon ATP binding.[18] The exchange of phosphate into ATP during the steady state ATPase of actoS-1 was a factor of 50 times faster than the exchange measured in the ADP state that is formed by addition of ADP to actoS-1.[326] This shows that state 8 is composed of at least two states, one of which A·M'D only has a significant population during the steady state hydrolysis of ATP. The release of products from the A·M complex is very much faster than from myosin alone, which accounts for the faster ATPase activity in the presence of actin.

Measurements of the energetics of the various steps of Scheme I provide insight into how this interaction might function in a working fiber. It is assumed that one cycle involves the transient association of a myosin head with actin resulting in the hydrolysis of one ATP molecule. The free energy of ATP hydrolysis in living frog muscle has been calculated to be -54 kJ/mol, although more recent estimates have been a little higher.[329,330] During isotonic contractions at velocities near maximum efficiency, more than 50% of the free energy utilized by the contractile proteins is converted into useful work. This suggests that during the powerstroke more than 30 kJ/mol is converted into mechanical energy. A reasonable hypothesis is to associate the powerstroke with a step, or steps in Figure 10 that involve a large drop in free energy and occur when myosin is associated with actin. The binding of myosin to actin, step 1→5, is very strong, $K = 10^8\ M^{-1}$, which has led to the suggestion that the free energy driving the powerstroke is derived from the formation of the actomyosin bond.[331-333] This reaction can be considered to be first order with a free energy change that depends on the effective concentration of actin seen by the myosin head. If one approximates this effective concentration by the actual actin concentration within the fiber, 0.5 m*M*, one obtains a free energy change upon complex formation of 27 kJ/mol, and if the effective actin concentration is higher more energy could be obtained. As suggested by the structural studies of rigor muscle, state 5 is a reasonable choice for the end of the powerstroke. Dissociation of this bond is produced rapidly by the tight binding of ATP to myosin with an association constant of about $10^{10}M^{-1}$.[334] Thus the energy associated with formation of the M·ATP bond, approximately 46 kJ/mol, is sufficient to dissociate the bond between actin and myosin. A synthesis of this information with the structural studies leads to a model for a hypothetical cross-bridge cycle. In this model the free energy for performing work is provided by the formation of the actomyosin bond, and the binding of MgATP provides the free energy to separate the actomyosin complex at the end of the powerstroke.[19,331,332,335] The hydrolysis of the bound ATP is required so that the products can be dissociated in a subsequent powerstroke. A more detailed account of the energetics of this model follows.

Starting in the cycle at the end of a powerstroke in state 5, the binding of ATP causes a rapid dissociation to state 2 (equivalent to the transition from configurations 4 to 2 in Figure 8). States 2 and 3 equilibrate and both are in rapid equilibrium with attached states, 6 and 7. States 6 and 7 are identified with configuration 3 in Figure 8. There is

some evidence that these weakly bound states, 6 and 7, have a structure that is quite different from the more strongly bound states, 5 and 8. The regulatory proteins inhibit the binding of S-1 and actin to form states 5 and 8 but do not affect the formation of states 6 and 7. This effect does not simply reflect the stronger bond formed in states 5 and 8 since the same results are obtained at a higher ionic strength where the association constant for state 8 has been decreased to that for states 6 or 7 measured at a lower ionic strength.[338] Thus, the weakly bound states, 6 and 7, differ both structurally and energetically from the strongly bound states, 5 and 8. An interesting analog of the weakly bound states is produced by cross-linking the two reactive sulfhydrals as discussed in Section IV.C.[339] The nucleotide-induced conformation change in the myosin head that determines its affinity for actin can be locked in by the cross-linking reaction. As discussed in Section V, states 5 and 8 appear to have similar structures, with the myosin heads relatively ordered and at an acute angle to actin, while the more weakly bound states, or their analogs, appeared to have a different structure that was more disordered. States 8 and 5 would be associated with configuration 4 in Figure 8. The transition from the weakly bound states to the strongly bound state 8 releases a considerable amount of free energy. In principle, chemical free energy could be converted into mechanical work in any of the states 5 through 8 in which myosin is complexed with actin. Work is performed by movement down a free energy gradient within a state, and not by the transitions between states. The complex between actin and myosin in state 8 has an affinity constant that is about 10^4 times greater than those of the weakly bound states 7 and 6, so that about 22 kJ/mol of free energy is available within state 8. The amount of work that could be performed by states 6 and 7 depends on the effective actin concentrations, and if one takes the actual concentration then little free energy is available for work in these states. The observation that fibers generate very little tension in the presence of ATP-γS also suggests that little work is performed in state 6.[261,361] This ATP analog is hydrolyzed slowly by the fiber so that the cross-bridge should be found predominantly in states 2 and 6 and if work were performed in state 6 it is reasonable to expect that the fibers should generate some tension. The release of ADP by acto-S-1 involves little change in free energy at the ADP concentrations found in living fibers (approximately 10 to 100 μM) so that free energy is probably not converted into work in state 5. Thus by the process of elimination, work is more probably performed by states 7 and 8. The exact energetics of these transitions are difficult or impossible to determine from studies done in solution, because they involve factors such as the effective actin concentration that most probably depend on the relative orientation of the myosin head and the actin site. While the above hypothetical cycle is reasonable and there is some evidence to indicate that it functions in the fiber, many details remain to be proven. For instance, there is no direct evidence that the free energy driving the powerstroke is derived from the formation of the actomyosin bond. The details of these interactions in the fiber are only beginning to be investigated. A few direct measurement of the kinetics of the contractile interaction within the filamentous array of the fiber have been made and are discussed below.

B. Interactions in Fibers

The traditional methods such as stopped flow, used to elucidate the kinetics of the contractile interaction in solution, are difficult to apply to intact fibers and investigations of the transient kinetics of cross-bridge interactions have resorted to new methodology including: photolyzable analogs of ATP, measurements of single ATP turnovers in myofibrils, and the effects of substrate variation on fiber contraction.

A comparison of the steady state rates of ATP hydrolysis in fibers and in solution suggest that the kinetics of the interaction of acto-S-1 resembles those of a fiber that is shortening at a relatively high velocity.[20,23] The ATPase activity of acto-S-1 from frog

extrapolated to infinite actin concentration, 4.5 s^{-1} is almost exactly that found for a frog fiber operating at maximum power output (both at 0°C). The isometric ATPase activity of frog muscle is much lower, 1.2 s^{-1}, reflecting the fact that substrate utilization is coupled to energy output. The energetics of rabbit fibers are less well explored, however, the isometric ATPase activity is of order 1 to 2 s^{-1}, compared to a maximum acto-S-1 activity of 20 s^{-1} (both at 20°C). Thus, either rabbit fibers have a larger differential between isometric and isotonic activities or the correspondence between solution and fiber is not as precise as in frog. For both frog and rabbit the Km describing the dependence of ATPase activity of isometric fibers on the ATP concentration is close to that of acto S-1 (both are about 15 μM).[20] In summary, the comparison of the steady state activities of the contractile proteins in solution and in fibers lend support to the idea that many aspects of this interaction in solution resemble those found in the fiber, but that some rates may be changed, e.g., those portions of the cycle occurring in the powerstroke may be slow in the fiber and fast in solution, and constraints imposed by filament geometry may influence rates of myosin associations with actin.

The most direct measurements of the kinetics of the contractile interaction within fibers have been obtained by photogeneration of ATP from an inactive precursor, "caged ATP".[340] ATP is introduced into a rigor fiber within 1 to 3 ms by photolysis. The ATP is cleaved rapidly with a rate constant in excess of 35 s^{-1}, suggesting that the hydrolysis step is probably similar to that seen in solution.[341] The initial ratio of the product, P_i, to myosin sites suggests that the free energy associated with the cleavage step is small as is also found in solution. The second order rate constant for the release of the myosin cross-bridge by the binding of ATP, approximately 10^6 M^{-1} s^{-1}, is similar to that seen in solution for the dissociation of acto S-1.[342] The rate of the release is faster when the initial rigor tension is higher, an important observation that is the first to directly confirm the common hypothesis that many rates in the fiber must depend on cross-bridge distortion. In the presence of calcium the rate of tension increase following photolysis gives a rate for the reattachment of released heads to form force-generating states.[343] This rate is rapid, around 100 s^{-1}, implying that transitions among myosin states detached from actin or among attached states that do not generate tension occur rapidly. The dissociation of myosin by ATP with subsequent hydrolysis and rebinding to actin all occur rapidly in solution as discussed previously, so that the rapid rise in tension can be explained by the known kinetics. In isometric muscle the ATPase activity per head, approximately 1 to 5 S^{-1}, is orders of magnitude slower than the measured rate for reattachment so that other steps must limit the rate of the cycle. Such steps could include an attachment reaction that is in rapid equilibrium as suggested by solution studies, along with slow isomerization steps among attached states.

Addition of small amounts of ATP to myofibrils, approximately stoichiometric to the concentration of myosin heads, provides another method of measuring kinetics in the filament array. Measurements of the increase in tryptophane fluorescence in such experiments show that it is similar to that obtained with acto-S1 in solution.[344] The ratio of bound ATP to ADP·P_i in myofibrils is approximately the same as that found for myosin in solution, showing that the free energy for hydrolysis ($K \sim 3$ under the physiological conditions employed) is not changed by the fact that the hydrolysis is occurring in the filament array of the myofibril.[345] Whether hydrolysis of ATP alters the configuration of an attached myosin head remains an important unanswered question.

Measurement of the exchange reactions that occur between water, phosphate, and nucleotides can be performed in fibers and in solution, and the results compared. In isometric fibers the amount of oxygen exchange decreases considerably when the fibers are activated, as expected because the rate of $7 \rightarrow 8$ is accelerated by the interaction with actin.[19,346] The pattern of exchange can not be fit with a single pathway so that

there must be at least two mechanisms for ATP hydrolysis in the active fiber. The origin of these mechanisms is not understood but may relate to geometrical constraints that vary the effective actin concentration seen by a myosin head.[326] The rate determined for the release of P_i, step 7 → 8, is sufficiently rapid that it must precede the rate-limiting steps in the overall cycle. Comparison with the data obtained in solution implies that the effective actin concentration is high.[20] The resynthesis of ATP from ADP and added P_i is also catalyzed by acto-S-1 in solution. This reaction occurs during steady-state hydrolysis of ATP from an A·M·ADP intermediate state that precedes the states obtained by simple addition of ADP to A·M. In fibers this exchange is accelerated and depends on the type of contraction, the exchange being faster in stretched fibers than isometric fibers and lowest in fibers that were shortening.[20,326,347] These phenomena are easily understood qualitatively in terms of models of the cross-bridge states. Because of steric constraints the transitions among the relevant states occur with little loss of free energy in the fiber and thus should be easily reversed, while in solution the transition from A·M·ADP·P_i to A·M·ADP would quickly result in a large drop in free energy which would make reversal more difficult. Although the results fit into a consistent picture qualitatively, it is surprising that the differences observed are so small, isometric fibers have an exchange rate only five times faster than acto S-1 and the difference between stretched and shortening muscle is only a factor of two. Additional evidence that phosphate binds to fibers is given by the observation that it accelerates the rate of tension decrease following photolysis of caged ATP.[348]

Another method of assessing the rates of reactions in the fibers is to measure the mechanics of contraction as the level of substrate is altered. Decreasing the concentration of ATP results in an increase in the isometric tension and a decrease in the maximum velocity of contraction.[302,349-351] These results are consistent with a model in which the binding of ATP is required to release the head from actin. If the rate of head release is assumed to be the same in fibers as is found in solution, a simple analysis of the data indicates that rapid binding of ATP occurs in a small region of X, approximately 2 nm.[349] At physiological levels of ATP the maximum contraction velocity is not limited by the rate of ATP binding, and is most probably determined by the rate of product release or by an associated isomerization step.[351] The measured rate of product release is consistent with this step having a role in limiting the maximum contraction velocity.[352] The addition of ADP to contracting fibers increases isometric tension and decreases the contraction velocity while the addition of phosphate decreases the isometric tension and has no effect on the maximum velocity of contraction.[353-355] These results are expected if ADP is released immediately prior to the binding of ATP and if phosphate is released at some point within the powerstroke.

In summary, the results obtained in fibers suggest that many similarities exist between the reactions occurring in a fiber and those in solution. The myosin head is dissociated rapidly from a rigor state by the binding of ATP. ATP is cleaved rapidly with little change in free energy. Myosin with bound ATP or products reattaches rapidly to actin with release of phosphate followed by release of ADP and return to a rigor state. In isometric fibers the rate of this cycle is slow and is probably limited both by slow steps within the powerstroke and by the rate of attachment of myosin to actin. This latter transition may be slow because myosin must wait for an actin to enter the region in which attachment is permitted, or it may be slow because attachment is a multistep process consisting of a rapid equilibrium followed by an isomerization to force producing states. A multistep attachment has also been proposed to account for the drop in power output at high contraction velocities.[356]

One goal of muscle biochemistry has been to describe the mechanics of muscle contraction in terms of the kinetics and energetics of the actomyosin interaction. The first such attempt was presented by Huxley in 1957, who showed that the mechanics and

energetics of steady state contractions could be explained by a simple and elegant model that described the rates of attachment and detachment of a cross-bridge from actin.[293] Subsequent workers have elaborated on this framework in order to explain the transient responses of muscle to step changes in load or length and to incorporate known biochemical intermediates. The thermodynamic formalism to analyze the connection between free energy and reaction rates has been developed.[335,357]

The known energetics of many intermediates in the contractile cycle have been used to construct the most biochemically explicit model of the cross-bridge cycle.[335] The relation between biochemistry and the muscle fiber is made by assuming that in solution, states relax rapidly to their free energy minima. The difference in free energy between the minima of two states in the fiber can be determined by measuring the corresponding difference in free energy between the two states in solution. A common assumption in many of these models is that a particular configuration of the cross-bridge corresponds to the occupancy of the nucleotide site on myosin, i.e., in a rotating cross-bridge model the angle of the myosin head is specified by the nucleotide bound to it. This may not necessarily be the case and a different class of models in which the nucleotide shifts the equilibrium between strongly and weakly bound states has also been proposed.[22]

A number of pieces of evidence lead to the conclusion that attachment of cross-bridges at the beginning and detachment at the end of the powerstroke are rapid processes. Fast transitions were invoked to explain the dependence of tension transients on the ATP concentration, and to explain the dependence of stiffness on the velocity in relaxed fibers at low ionic strength.[239,301,302] The binding of myosin to actin in solution may also be a multistep process starting with a rapid equilibrium between bound and unbound states.[318] Together these observations suggest models in which transitions between bound and unbound cross-bridges occur via rapid equilibria. Because less free energy is lost in making such a transition it would lead to greater efficiency of energy transduction and should be considered as a likely candidate in future models.

To date, model building has involved making fairly arbitrary assumptions concerning the nature of the cross-bridge states and then simulating the expected fiber response by using numerical methods to solve the set of linked differential equations. The rates between states are assumed to vary with the relative distortion, and the dependence of rates on x can not be easily determined from the solution studies. Although the rates of many reactions are assumed to vary with cross-bridge distortion, the investigations that have measured such effects have in fact found little variation.[326,347,358] The number of independent parameters required to specify even a simple model is large, however, a considerable body of data on the transient and steady state responses are now available to constrain such models, and no model has attempted to fit all of these data. As more exact measurements of cross-bridge kinetics are made in active muscle fibers the number of constraints imposed should approach the number of independent parameters and it should become possible to attain the above goal.

VII. SUMMARY

Although the mechanism of contraction is not yet understood at a molecular level, there has been considerable progress towards this goal in recent years. Together the studies reviewed above suggest a model in which a myosin head binds weakly to an actin filament, a conformational change in the protein complex occurs translating the actin filament by 5 to 10 nm, ending in a tight complex between actin and myosin with the myosin forming an acute angle with the actin. A number of crucial questions remain unanswered.

One question of fundamental importance is the nature of the conformational

changes occurring within the powerstroke. Several years ago the mechanism of cross-bridge action in fibers appeared to be better understood than it is today. Data obtained using electron microscopy and X-ray diffraction first led to a model in which a cross-bridge attached at an angle of 90° and executed a powerstroke by changing orientation to 45°. More recently, data obtained using probes sensitive to orientation have indicated that at least a portion of the myosin head does not change orientation during the powerstroke, so that the original view of a relatively rigid rotating cross-bridge is not correct, leading to a consideration of a number of other models. Some information on the events within the powerstroke may be obtained from the identification of states in which myosin appears to be weakly bound to actin. These states are good candidates for those at the beginning of the powerstroke, and they fulfill the long-sought-for goal of finding two different states for myosin attached to actin. The orientation of the cross-bridge in these states appears to be more disordered than in the rigor state. However, a specific orientation has not been determined. Although a number of models of cross-bridge action have been proposed, the most plausible one appears to involve a change in the orientation of a portion of myosin that is distal to actin while a second portion proximal to actin remains rigidly fixed to the actin filament. This model is supported by considerable but not conclusive evidence, and other possibilities should also be considered.

A second problem, whose solution is pertinent to the question raised above, concerns the location of the active elements responsible for force generation. A number of experimental results suggest that a single head of a myosin molecule, interacting with an actin filament, can generate tension. Some data have led to the conclusion that the myosin portion of the mechanism is found in the head region, however, this conclusion is not unequivocal. Monoclonal antibodies offer one route to identifying the location of these elements. The traditional genetic approach of identifying the phenotype associated with known mutations has been difficult to apply to the contractile system in the past. However, the cloning of the genes for both actin and mysoin, along with advances in the area of site specific mutagenesis, may now make this a productive approach.

Another approach that could answer both of the above questions is the measurement of protein conformational changes occurring in solution that could be identified with force production. Although considerable effort has been expended in this direction, to date, no changes in conformation have been unambiguously linked to function, leading to the conclusion that either these changes are subtle or that the active states have not been reconstituted in solution.

Although measurements of the kinetics and energetics of the interaction of actin, S-1, and ATP are a major determinent of the above hypothesis a number of areas of uncertainty remain. These interactions have been relatively well characterized in solution, where the data suggest that both S-1 · ATP and S-1 · ADP · P_i form a weak attachment to actin, that the hydrolysis of ATP occurs with little change in free energy independent of attachment to actin, and that the release of products leads to a strong bond with actin. The next step in these investigations is to elucidate how these interactions are altered when they occur in the filament array of the fiber. Initial experiments have suggested that the cycle of events in the fiber has many similarities with that in solution. It is generally expected that the rate of many reactions should depend on the relative positions of a myosin head and the actin with which it is interacting. Surprisingly, the investigations that have searched for such a dependence have found little or no effect. A number of questions remain before the kinetic studies can be correlated with structural information. A popular hypothesis, that the orientation of the myosin head is determined by the nature of the nucleotide bound to the myosin, remains unproved. There is also no evidence that the free energy driving the power-

stroke is derived from the formation of the actomyosin bond. These and other questions will have to be answered before more detailed models of cross-bridge kinetics can be formulated.

Elucidation of the molecular mechanism of force production will necessarily require knowledge at high resolution of the tertiary structures of the proteins involved. Steady progress towards this goal has been made. The primary structures of all the protein components have been determined and both actin and the myosin head have been crystallized. The structure of actin should be known to high resolution within a short time, while that of the myosin head will take at least several years. A synthesis of this structural knowledge with information on protein dynamics obtained by a variety of other techniques should eventually attain the above goal. If the progress towards this goal appears halting it is because the difficulties are great. There are a number of energy-transducing molecular machines in biological systems, e. g., ion pumps, chloroplasts etc., and to date, none of these is understood at the molecular level. The studies of the mechanism of force production may be as close to this goal as are the investigations of these other systems.

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