MECHANISM OF ACTOMYOSIN ATPASE AND THE PROBLEM OF MUSCLE CONTRACTION

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INTRODUCTION

All organisms are capable of converting chemical potential energy into useful work. How it is done is still somewhat a mystery despite progress that has been made in the last 25 years. Muscle contraction, various kinds of cellular locomotion, and flagellar movement are considered to be related in that they all depend on some variation of the sliding filament mechanism. Active transport presents a similar problem except that the work is done against a gradient in chemical potential rather than mechanical potential energy.

In each case, the essential requirement is the coupling of a chemical reaction, generally the hydrolysis of ATP, to a series of conformation changes of an asymmetric structure. The two systems most amenable to detailed investigation are the muscle contraction and ion transport systems. Recently, a general theoretical method for handling coupled reactions was developed which is applicable to either system.[75-77] It is hoped that these systems can be understood in sufficient detail to allow general conclusions to be drawn about energy-coupling mechanisms. Perhaps the most interesting generalization to come from recent studies on motility is that the actomyosin system is ubiquitous.[169] Cell movement and its control by internal or external factors are central problems in biology which may be untangled with the muscle mechanism as a guide.

This review will concentrate on the mechanism of actomyosin ATPase and its relation to the mechanism of muscle contraction. Relevance to other motile mechanisms will be included only incidentally. Some aspects of the problem have been treated in recent reviews by Trentham et al.,[221] Mannherz and Goody,[123] who include a clear discussion of the use of substrate analogues in the study of cross-bridge orientation, Tonomura and Inoue,[218] who present a somewhat different interpretation of the kinetic evidence, and Squire[201] who has given a clear presentation of the structural evidence.

STRUCTURE AND FUNCTION OF MUSCLE

General Properties of Muscle

The objective of chemical and structural studies is to understand how ATP hydrolysis can produce macroscopic movement. The major advantage in using muscle as an example of a motile system is its very regular yet relatively simple structure. A first approximation of the contractile mechanism was almost self-evident when the main features of muscle structure were resolved.[89,90] The functional unit of striated muscle, the sarcomere, consists of two sets of interdigitating filaments which move past each other during contraction — the sliding filament mechanism.

The thick filaments have regularly spaced projections or bridges consisting of the globular portion of the myosin molecule, including its ATP and actin binding sites. The thin filaments are made up primarily of actin. Myosin has a low ATPase activity under physiological conditions (0.15 *M* KCl, 5 to 10 m*M* $MgCl_2$, pH 7) but can be activated tenfold by a stoichiometric amount of actin. Actin and myosin form a complex in solution which is largely dissociated by ATP. These facts, available 20 years ago, can be assembled into a preliminary mechanism with some confidence that one is on the right track. Myosin projections from the thick filament interact with actin residues of the thin filaments, and a relative sliding movement is driven by the hydrolysis of ATP.

It is worthwhile to stress the importance of these earlier results. The structure of a muscle speaks for itself in a way that the structure of a cilium or a biological membrane cannot. The former has too much structure and the latter too little to provide a clue to the function. In muscle, it was reasonably clear that the coupling mechanism must involve conformation changes of the myosin cross bridges through mutual interactions with actin and ATP. It could also be guessed that the main difference between a muscle at a long length and one at a short length is that the latter is shorter, i.e., the cross bridges undergo a cyclic process of attachment and detachment, exerting a force through a small distance in each cycle. In 1957, A. F. Huxley[83] combined these observations in a simple model that accounted for many of the properties of muscle. His model represented a major step in the solution of the problem because it showed how to construct a theory relating the macroscopic properties of muscle (force, velocity, and heat production) to its structural and biochemical properties. Our knowledge of the system has been considerably refined in the past 20 years, yet a 1977 model would have much in common with the 1957 one.

The topic of this review is the ATPase cycle of actomyosin, but its significance can only be appreciated in terms of the presumed cross-bridge cycle of the muscle. The relevant information on muscle structure will be summarized first. It seems particularly perverse to suppose that the structure of muscle and the enzymatic properties of actomyosin have little to do with the mechanism of contraction; therefore, abstract models which ignore these detailed phenomena will not be discussed. It is assumed that muscle contraction is understandable in terms of enzyme kinetics suitably modified to take into account the constraints introduced by the lattice. The problem is solvable in principle if not yet in practice.

Muscle Structure

The objective of structural studies is to determine the most probable trajectory of the cross bridge in the cycle. In 1969, H. E. Huxley[85] reviewed the evidence and presented a simple structural model of the cycle which became the basis of all subsequent discussions (Figure 1). The essence of the model is a preferential attachment of the cross bridge at some angle θ_1, a rotation through the angle θ_2-θ_1, and a detachment at angle θ_2. In the diagram, the angles are 90 and 135° with respect to an axis in the thick filament which points toward the end of the sarcomere. A relative translation of about

100 Å is expected because the length of the globular head is 100 to 150 Å. Perhaps the diagram unintentionally emphasizes a one-way cycle. The model is consistent with many general features of muscle structure and chemistry:

1. The only connections between thick and thin filaments are the cross bridges; because there is no change in length of the thick and thin filaments in a contraction over the physiological range, it is clear that the bridges both generate and sustain tension.
2. The tension beyond rest length is proportional to the filament overlap[56] and, therefore, to the number of bridges potentially capable of interaction with the thin filament.
3. The globular head is connected to the thick filament backbone via a coiled-coil helical segment which can bend outwards to accommodate the variable interfilament space.
4. The actin and myosin form a complex in solution which is the counterpart of attachment in muscle.

Models based on a moving cross-bridge cycle will differ from Figure 1 in their choice of the number of states (four in Figure 1), the angles θ_i, and the incorporation of mechanical elements to take up the tension ("springs"). In the original model, the attachment angle θ_1 was based on an analysis of the diffraction pattern of resting muscle.[88] This model suggested that the cross bridges are perpendicular to the thick filaments and that the ends do not extend to the actin radius. It was assumed that the cross-bridge orientation in resting muscle corresponds to state 1 in the active cycle and that the cross bridge simply swings out and attaches in the same orientation in state 2. The cross-bridge orientation in state 3 was inferred from the rigor diffraction pattern, the orientation of the single heads of myosin subfragment-1 (SF-1) in the actin-SF-1 complex,[143] and the orientation of cross bridges seen in electron micrographs of insect flight muscle in rigor.[173] The resting and rigor diffraction patterns differ in the relative intensities of their equatorial reflections arising from the hexagonal lattice of thick filaments (1,0 and 1,1 reflections). These shifts in intensity were interpreted as a movement of mass from the vicinity of the thick filaments to the position of the thin filaments at the trigonal points of the lattice. Electron micrographs also showed a decrease in the relative amount of material associated with thick filaments in the rigor state.[84] In addition, in rigor as compared to resting muscle there was an increase in the intensity of reflections belonging to the actin periodicity (355 to 385 Å) and a loss of intensity of orders of the head periodicity (429 Å). This provides even further evidence for attachment of the heads to the actin filament in rigor.[65,67,141]

The results of recent structural studies may require revisions of the details of the Huxley model, but discussions have remained within the context of a moving cross-bridge cycle. Agreement has not yet been reached on the packing of myosin molecules in the thick filament, as reviewd in 1975 by Squire.[201] The symmetry of the structure cannot be obtained directly from the diffraction pattern. The spacing between sets of cross bridges is 143 Å, but two, three, or four bridges could occur at each level.[200] The cross-brdige positions could be described by a 6/2 (two helices with six cross-bridge units per turn), a 9/3, or a 12/4 structure. The mass ratio of myosin to actin increases with the number of helices so that biochemical evidence is probably consistent with the 9/3 or 12/4 structure. A 6/2 symmetry has not been disproven, but it would require two myosin molecules on each point of the helical lattice. Based on the molecular weight of a thick filament, four myosins per level are favored by Morimoto and Harrington.[146]

The diffraction pattern of resting muscle can be calculated for possible models, but it is necessary to specify the helical symmetry, the length and shape of the cross bridge,

the tilt angle Ψ, the azimuthal angle ϕ, and the relative placement of the two myosin heads to one another. It is not clear whether there is a unique solution to this problem given the present uncertainty in the parameters. The orientation of the cross bridge is uncertain, but there appears to be agreement that $\Psi = 90$ and $\phi = 0$, the original Huxley model, do not fit the pattern. A structure in which the cross bridge is tilted away from the normal and twisted ($\phi \neq 0$) angles is a possible solution.[66,172] In invertebrates (horseshoe crab, lobster, scallop), the layer lines are not sampled by the hexagonal filament lattice, which simplifies the comparison of the calculated and observed patterns. Wray et al.[244,245] propose a tilted and twisted cross-bridge model for these muscles. Treatment of thick filaments with chemical cross-linking agents introduces links between head and rod regions at rest or in rigor[204] which is consistent with a twisting of the head across the surface of the thick filament.

Thus, there is no evidence that the orientation in state 1 is 90°. In any case, the assumption that the orientation of the cross bridge in the relaxed state determines the preferred angle of attachment in the active cycle may not be justified. The rotational relaxation time of a myosin head of a thick filament is approximately 1 μsec,[212] while tension development or change in intensity of the equatorial reflections occurs on a millisecond time scale.[87] A model requring the reorientation of a cross bridge before binding to actin could be accommodated by the available evidence.

The interpretation of the change in intensity of equatorial reflections (1,0 and 1,1) has also been debated.[68,119] The problem arises from the uncertainty of the length and orientation of the cross bridge in the relaxed state. If the cross bridge extends out to the radius of actin, attachment could be an azimuthal rotation. If the cross-bridge density is well within the actin radius, attachment could be largely an outward motion of the stem as in the original Huxley model, a change in tilt, a change in azimuth if it is twisted across the thick filament, or a combination of all three.

The evidence for a 135° angle in rigor has been strengthened by the reconstruction of micrographs of the actin-SF-1 complex[143] and the actin-tropomyosin complex[229] and the analysis of the rigor diffraction pattern of insect flight muscle.[141] However, layer lines of the myosin periodicity are absent in the rigor pattern[65] and the deduction of the structure is dependent on model building.

If Figure 1 or something like it is a realistic picture of the cross-bridge cycle, one might expect the diffraction pattern of a muscle in isometric contraction to consist of reflections which arise independently from the four cross-bridge states and are weighted according to the relative occupancy of the states. Unfortunately, the diffraction pattern consists of layer lines similar to the resting state (429-Å periodicity) but with 30% of the resting intensity, a meridional reflection at 144.8 Å as in the rigor pattern, and no "rigor-like" layer lines. The intensity changes which are relative to the relaxed pattern do not depend on the extent of overlap of the thick and thin filaments. If the decrease in intensity of the layer lines or the change in intensities of lattice reflections (1,0 and 1,1) is taken to be a measure of the movement of the cross bridges from their position close to the thick filament to the vicinity of the thin filament, then about 45% of the cross bridges have moved outward in frog muscle and 10 to 20% in insect flight muscle.[5] Yagi et al.[246] have recently reported that 80% of the cross bridges have moved into the actin region in frog muscle. The change in spacing of the meridional reflection from 143.4 to 144.8 Å may indicate a change in the structure of the thick filament so that the detached cross bridges are not in the well-ordered state of the relaxed muscle. Thus, the orientation of the cross bridges in the detached states (1 and 4 of Figure 1) is not specified. The absence of rigor-like layer lines, attributed to the attached bridges in the 135° orientation (state 3), indicates that if this state occurs in the cycle, only a small fraction of the bridges are in state 3. It should be noted that the intensity of a reflection is proportional to the square of the fractional occupancy

of the state. Thus, if 10% of the bridges were in state 3, the intensity relative to the rigor pattern would be approximately 1% which is not detectable.

The diffraction pattern of the excited muscle does not prove that there are two attached states with different orientations, and a demonstration of this point is crucial for moving cross-bridge models. Other approaches to this problem using analogues of ATP will be discussed in a later section. Methods which detect a signal proportional to the number of elements in a particular orientation are needed, and an alternative method has been explored by Morales and collaborators.

A fluorescent label (IAEDANS) can be fixed rigidly to the myosin head and the orientation determined relative to the long axis of the head. The average orientation of the heads in different states, resting, isometric contraction, or rigor may be calculated from the degree of polarization. Although this method is restricted to glycerinated or skinned fibers, it may not be a serious limitation.[39,155,156]

Results obtained by this method are slightly different than those obtained by X-ray diffraction. The polarization for fibers stretched beyond overlap was the same for all three states: resting, contraction, and rigor; while the X-ray pattern showed partial or complete loss of the layer lines derived from the highly ordered resting state when muscle is excited or in rigor, respectively. The polarization for contracting and rigor states increased with the degree of overlap, and the change was larger for rigor as compared to active muscle. In the X-ray case, the loss of the relaxed pattern was independent of sarcomere length and, thus, a cooperative effect. In a recent study[17] the polarization in rigor agreed with the calculated value for a 135° angle of the cross bridges although the polarization for relaxed fibers corresponded to a random orientation of cross bridges. The differences in the results of the polarization method as compared to X-ray diffraction might be explained if the ordered state, which gives rise to the set of layer lines indexing on a 429 Å repeat, is already lost in the glycerinated fibers. The change in polarization relative to the resting state arises from the orientation of the cross bridges attached to thin filaments. Another alternative is that the cross bridges swing out at a constant angel of tilt on activation and the angle is changed if the cross bridges can interact with actin. The polarization method promises to be a very valuable one for studying cross-bridge orientation in the attached state. Thus, the moving cross-bridge model is a plausible mechanism consistent with much of the evidence on muscle contraction but one which still lacks definite proof.

Structural Studies of Ca Regulation

The primary event in excitation of all muscles is the release of calcium from the sarcoplasmic reticulum or cell membrane, but there are at least three mechanisms by which the contractile system is turned on and off by calcium. In the first type, the calcium acceptor is troponin, a protein bound to the thin filament. Regulation involves a change in structure of the thin filament. Type 1 includes vertebrate skeletal (striated) and cardiac muscles. In the second type, the calcium acceptor is a polypeptide chain which is part of the myosin molecule, the P-light chain. Type 2 regulation is widely distributed among invertebrates. In the third type, the acceptor may be a protein kinase which is specific for the P-light chain of myosin. Phosphorylation of the protein is necessary for activation and dephosphorylation by a phosphatase for relaxation. Vertebrate smooth muscles belong to this class of regulation. One example of a nonmuscle actomyosin, the enzyme from platelets, appears to be activated by a phosphorylation mechanism.[2] However, Ebashi et al.[42] have obtained evidence for the regulation of smooth muscle by yet another protein factor. The chemical properties of these various systems will be discussed in a later section.

The structural basis of regulation has been most extensively studied for type 1. The thin filament consists of actin, tropomyosin (TM), and troponin (TN) in a mole ratio of 7:1:1, respectively. Tropomyosin is a complete helical-coiled coil with a length of

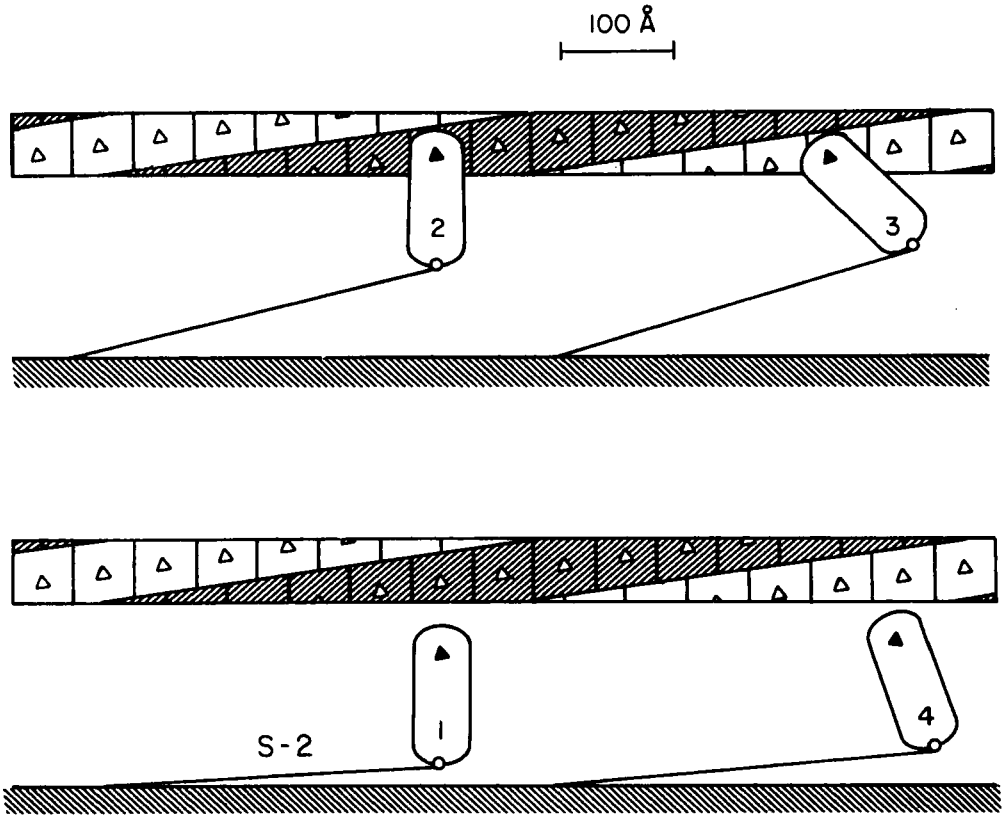


FIGURE 1. The cross-bridge cycle (after Huxley, H. E., Huxley, A. F., Pringle, J., and others). Two myosin molecules are shown (only one head is drawn) interacting with the same actin filament. The diagram is approximately to scale in order to illustrate the geometric constraints of the lattice. The S-2 region of myosin, the coiled coil connecting the head to the thick filament, is slightly longer and also thicker than shown here. The thin filament is drawn as a 28/13 helix with 14 residues per turn and a pitch of 385 × 2 Å. The origin of the S-2 region has a spacing of 429 Å. The radial distance from the surface of the thick filament to the central axis of the thin filaments varies with muscle length and here is 190 Å. Binding sites on actin residues are shown as △ and binding sites on myosin as ▲ in state 1 and as ◀ in state 4 to illustrate the assumptions that there are two myosin states which bind to actin at different angles and that the transition between attached states is asymmetric, i.e., here the transition produces a counterclockwise rotation. The bound bridge is drawn as lying on top of the actin (see Reference 15 and Figure 2). The cycle of transitions of 1→2→3→4→1 can be read as the successive steps for a single cross bridge to illustrate the cycle. The orientation angle of the cross bridge in a detached state is probably not fixed nor correlated with the state. State 1 may be a myosin-product intermediate and state 4 a myosin-substrate intermediate (Lymn-Taylor model). The figure may also be read as two pictures of a pair of cross bridges; the first undergoes a transition from state 1 to 2, the second from state 3 to 4. It is evident that a suitably oriented actin site may not be accessible to a cross bridge for some values of the relative translation of the two lattices. Also, attachment requires some distortion of the globular head or S-2 region unless the binding sites are directly opposite. The cross bridge in state 2 has attached in the most favorable orientation. The cross bridge in state 3 has presumably attached two residues to the right of its present position, which required some distortion of the structure. The diagram does not imply a correlation of attachment or detachment steps of different bridges.

In the Huxley-Simmons model, the transition of 2→3 occurs in more than one step and the distortion at constant sarcomere length is illustrated by a spring plac in the S-2 region. In the Eisenberg-Hill model, the distortion is included in the potential energy surface of the interacting sites and is not easily drawn. The concept is roughly illustrated by representing the conformation change of myosin as ▲ to ◀ which is compensated for by a 45° counterclockwise rotation of the head. Thus, the combined conformation change and rotation leaves the orientation of the binding site unchanged.

395 Å which follows the contour of each actin helix in a position partway into the groove (Figure 2).[229] Troponin is bound to tropomyosin and also somewhat weakly to actin with a periodicity of 385 Å. Pure actin has a half-period repeat of 355 Å and is referred to as a 13/6 helix. (The notation is different than that used to describe the thick filament. Actin consists of two helices with 13 residues per turn related by a 180° rotation and a translation of one half a G-actin residue. The structure is also described by a single helix with 13 residues in six turns.)

A model of regulation, the tropomyosin-shift model,[64,86] was proposed based on the following observations. Optical reconstruction of the actin-tropomyosin complex places the TM just outside the region occupied by myosin subfragment-1.[229] In a complex of actin, tropomyosin, the inhibitor subunit (TN:I), and the tropomyosin binding subunit (TN-T) of troponin (an inhibited form of actomyosin ATPase) the tropomyosin density occupies a position farther out of the groove toward the SF-1 binding site (or the tropomyosin density appears to have merged with the actin subunit). These observations led to a simple mechanism in which a shift in the position of tropomyosin blocks the attachment of myosin (Figure 2). Similar conclusions can be drawn from a comparison of the X-ray diffraction pattern of relaxed and rigor muscles.[64,86,163,222] The second-order reflection of the actin helix (180 to 200 $Å^{-1}$) is enhanced in rigor. A calculation using a sphere and rod model to represent the actin-tropomyosin structure predicts the change in intensity from a movement of tropomyosin further into the groove in rigor.

Although the tropomyosin-shift model is consistent with structural studies, there are some reservations as to whether it can account for all of the biochemical evidence described in a later section. An alternative description is that of a change in the orientation or conformation of the actin subunit, as was recognized by the proponents of the tropomyosin-shift model. At the least, it is necessary to consider changes in actin structure in the relaxation mechanism. In relaxed frog muscle, the actin reflections do not fit very well to an integral helix with 13 residues per turn.[88] The actin reflections in insect flight muscle in rigor fit a helix with 14 residues per turn with a half-period repeat of 385 Å (a 28/13 helix in the present notation because there are 28 residues in 13 turns of a single helix).[141] The actin half period is also 385 to 390 Å in scallop adductor muscle[142] and some other invertebrate muscles.[244] The troponin repeat of 385 Å and the 7:1 of G subunits to troponin and tropomyosin fits better with a 28/13 helix. However, in intact muscle there is no evidence that the pitch of the actin helix changes in the transition from rest to active or rigor states.

A study of paracrystals and X-ray diffraction of oriented actin[53,157] established the following helical parameters: pure actin, 13/6; actin-TM, 28/13 (in later studies[158] paracrystals of actin-TM with either 13/6 or 28/13 structures were obtained); actin-TM-TN, 13/6; and actin-TM-TN plus Ca, 28/13. The Ca-dependent transition occurred over a range of Ca concentrations in which muscle or actomyosin ATPase is activated. These studies provide evidence that regulation may involve a change in actin pitch, and furthermore, in the two types of actin-TM structures with different pitch, the radial position of tropomyosin is unchanged. The tropomyosin-shift model explains most of the evidence, but there may also be a change in conformation of actin residues.

In molluscan muscle which contains no troponin and is regulated by Ca binding to myosin, a similar change occurs in the actin reflections but the intensity ratio is smaller.[163,222] These same arguments applied to the vertebrate muscle would predict a tropomyosin shift upon activation. It would then be necessary to suppose that this alteration was caused by interaction with myosin, as noted by the authors, which is to say that molluscan muscle turns itself on — the initial association of myosin states containing bound Ca alters its thin filament structure. The model also requires tropomyosin to be in the blocking position in relaxed muscle, raising the question of whether this conformation is stabilized by some protein other than troponin.

A further problem was raised by the observation that the ordered structure of cross bridges in relaxed frog muscle is essentially lost on activation and that the cross-bridge spacing increases from 143.4 to 144.8 Å.[65] Since these changes are seen even when the sarcomeres are extended beyond overlap where the thick filament cannot interact with actin, then Ca released on excitation may also alter the thick filament structure. The change in spacing could be explained as a change in pitch of the helix from 9/3 to 8/3, as suggested by Haselgrove. If we suppose that in relaxed muscle the ordered state of the cross bridges results from some interaction which locks the bridges down, then excitation could involve an "unlatching" of the bridges in response to Ca binding. This alteration could be accompanied by a change in the helical pitch of the thick filament.

Some auxiliary evidence appeared to support this model. As discussed in a later section, the P-light chain of myosin has a Ca binding site although the dissociation constant (10 μM) is rather high compared to the concentration needed to activate muscle or actomyosin ATPase (1 μM). Magnesium also competes with this Ca site at concentrations of 1 mM, and the free Mg concentration is probably 5 mM in muscle.[21] In addition, striated muscles contain large amounts of the Ca binding protein, parvalbumin,[109] and it is already difficult to explain why troponin can successfully compete against parvalbumin for Ca. Measurements of the sedimentation constant of thick

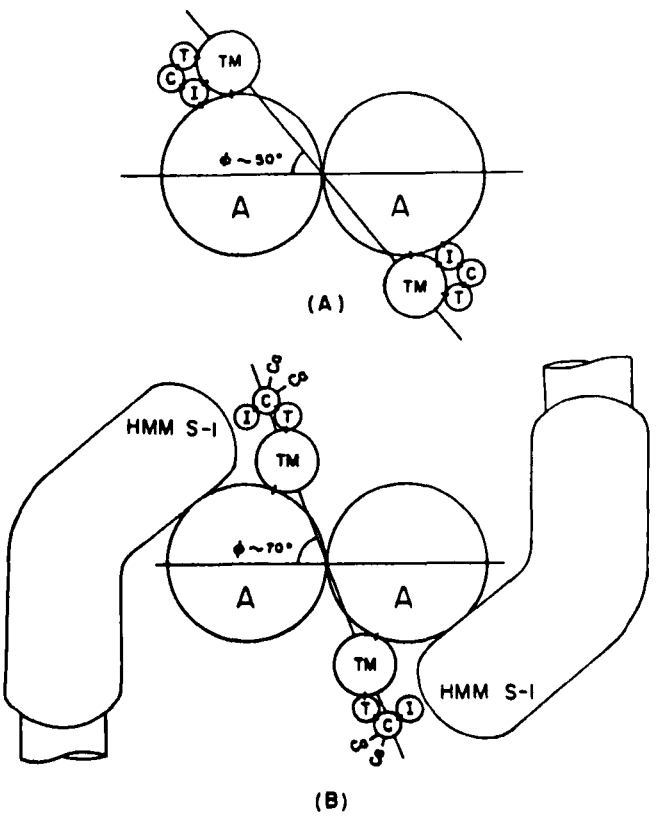


FIGURE 2. A model of type 1 regulation. A. Relaxation in the absence of Ca^{2+} B. Activation. A, actin; TM, tropomyosin; T, TN-T I, TN-I; and C, TN-C. Interactions between proteins are indicated by a short connecting line. (Reprinted with permission from Potter, J. D., and Gergely, J., *Biochemistry*, 13, 2702, 1974. Copyright by the American Chemical Society.)

filaments showed a 2% increase on Ca binding which could be interpreted as an unlatching of cross bridges.[146] But, this interpretation was not supported by Mendelson and Cheung,[138] who observed no change in the polarization of fluorescence of a label attached to the myosin heads. Sutoh and Harrington[204] have since shown that the cross-linking of the myosin heads to the filament backbone is not altered by Ca as might be expected from an unlatching mechanism. The change in sedimentation constant may have been caused by a small increase in pH since the freedom of rotation of cross bridges is markedly increased by raising the pH to the 6.8 to 7.5 range.[138] These results also call attention to the lability of the ordered structure of the relaxed muscle and the possible dangers in drawing conclusions from studies of reconstituted thick filaments.

Another possible explanation of the X-ray diffraction evidence is a cooperative change in the thick filament lattice produced by a small amount of overlap in some sarcomeres.[86] But, this argument still implies that the cross bridges can be released from the ordered state when there is no actin to receive them although the cause is not Ca binding.

To date, there is no strong evidence that Ca binding to the thick filament functions as a further control in the activation mechanism in vertebrate muscle. But, the ordered structure, giving rise to the layer lines of the relaxed pattern, is labile, and there is no evidence that the cross bridge in the detached states of the cycle has returned to the orientation of its relaxed state. The relaxed structure is not regained until a few seconds after the tension has fallen to zero.[86,246] Laser light scattering also detects some motion of the filaments after relaxation is complete when measured by the decay of tension.[24]

Mechanical Properties of Muscle

A detailed discussion of the mechanical behavior is outside the scope of the present review, but some evidence bears directly on the contractile model. Generation of tension by a muscle at constant length requires the stretching of some internal elastic element. Studies of the transient response to very fast stretch or release identified the elastic element as the cross bridge itself.[91] A decrease in length of 40 A per half sarcomere reduces the tension to zero, which is a rough measure of the half-width of the range of the movement between tension-generating states. The mechanical behavior can be represented by the adding of extensible elements to the cross bridges in Figure 1. Since the model is intended only as a plausible description, the placing of the spring at the base of the globular region of the cross bridge is satisfactory for a simple illustration. The transition between two attached states at constant sarcomere length stretches the spring. The muscle tension is proportional to the fraction of cross bridges in the stretched state. To account for the actual tension, it may be necessary to introduce more than two attached states[91] or allow for a wide distribution of attachment angles.[46]

Summary

In the relaxed state, the cross bridges are arranged in a regular structure in which individual cross bridges are probably tilted away from the normal to the thick filament and possibly twisted across it. The bridges are not in contact with the thin filament, although the radial extent of the bridges is uncertain. Excitation is triggered by Ca release which alters the structure of the thin filament (type 1) or the conformation of the myosin head (type 2 and 3), which may in turn affect the structure of the thin filament. The packing of cross bridges in the thick filament is probably not directly affected by Ca. Interaction between bridges and the thin filament occurs upon activation, but the behavior may not be a simple, independent binding of bridges. The interaction of a fraction of the bridges with thin filaments may disrupt the regular structure of the thick filament and may also alter the structure of the thin filament in type 2 and 3 regulation. Thus, activation may be a cooperative process in which the initial interaction of a fraction of the cross bridges facilitates the interaction of the remainder.

In the excited state, only a fraction of the bridges are attached to thin filaments (50 ± 30%), but there is no positive evidence that the detached bridges return to the resting orientation. The resting orientation also need not specify the most probable attachment angle. The X-ray evidence does not prove that there are two attached states wth different angles of attachment. The minimum energy configuration in the absence of ATP is at an attachment angle of 135°, but there is no proof that an appreciable fraction of the bridges is in this orientation in the cycle. The model drawn in Figure 1 may be a plausible description of the most probable orientation states, but the angles θ_1 and θ_2 are not specified.

BIOCHEMISTRY OF ACTOMYOSIN

The Biochemical Problem

In order to relate the structural model to a biochemical mechanism, it is necessary to consider those states defined by a preferred orientation angle θ_i to be discrete states in the usual chemical sense, i.e., that each state corresponds to a definite molecular structure which is separated from other possible conformations by an activation energy barrier. A chemical description of the mechanism will then consist of determining the intermediate states in the hydrolysis of ATP by actomyosin and finding the most probable pathway. The mechanism will involve at least four steps (as in Figure 1) and probably more, consequently, there are multiple closed paths or cycles which lead to the hydrolysis of ATP. The flux of ATP hydrolysis will hopefully be determined largely by one particular cycle which corresponds to the most probable cross-bridge trajectory in the mechanical model. One needs to establish a one-to-one correspondence between the biochemical states and the states of the cross-bridge cycle.

A limitation of the biochemical approach must be clearly stated. The determination of the intermediates and the rate constants of the biochemical mechanism has to be carried out in homogenous solution in order to analyze the kinetic data. The dominant pathway in solution may not be the most probable pathway in muscle because of the much higher effective concentration of the proteins and the coupling to the lattice constraints which will alter the rate constants of the transitions. In drawing conclusions about the mechanism of contraction, some caution and common sense are required. The extension of biochemical studies to ordered structures (myofibril suspensions and single fibers) is necessary once the solution mechanism is properly understood.

Enzyme Kinetics

Certain important properties can be determined by steady-state kinetic studies of myosin and actomyosin ATPase, but transient measurements have been the main source of information on intermediate states. A critical evaluation of the evidence requires some familiarity with kinetic analysis. As an example which illustrates the essential features, consider the following reaction which happens to apply to subfragment-1 ATPase:

$$M + T \overset{(1)}{\rightleftharpoons} M \cdot T_1 \overset{(2)}{\rightleftharpoons} M \cdot T_2 \overset{(3)}{\rightleftharpoons} M \cdot D \overset{(4)}{\rightleftharpoons} M + D$$

where T and D refer to the substrate (ATP) and products (ADP plus P_i) of the reaction, respectively. Upon the mixing of enzyme and substrate (in 1 to 5 msec), the concentrations of intermediates change with time until a steady state is reached. In the example, the reaction is sequential and there are four states: M, two substrate states, and one product state. Ideally, to determine the rates of all transitions between pairs of intermediates, one needs to have a signal proportional to the concentration of each intermediate. In practice, more than one intermediate may contribute to the amplitude of

a particular signal and some intermediates may be "silent." It is important to use as many different signals as possible. Actomyosin studies have utilized the following: intrinsic fluorescence of protein tryptophan, absorption at 290 nm, fluorescence or fluorescence polarization of bound labels, light scattering (for actomyosin association), proton release or absorption coupled to a pH indicator, conductance and direct measurements of ADP or phosphate formation.

A solution of the rate equations is available for the special case in which [T] remains constant during the transient and the inhibition by-products (the reversal of step 4) can be neglected.

The four states of the enzyme are denoted by X_1 through X_4, the rate constants for each step by k_i and k_{-i}, and the equilibrium constants by $K_i = k_i/k_{-i}$. The total concentration of enzyme Mo is $Mo = \Sigma X_j$, and the equilibrium constant K_t for the reaction $T \rightleftharpoons D$ is related to the K_i by $\pi K_i = K_t$, where π stands for product. The time dependence of intermediate concentrations is

$$X_j = C_j + \sum_{i=1}^{N-1} A_{ij} \exp(-\lambda_i t) \qquad (1)$$

where N is the number of states (four in this example). The solution is a sum of exponential terms, the number of terms being one less than the number of states. C_j is the steady-state concentration of X_j, and the A_{ij} can be determined from the initial conditions. The apparent rate constants λ_i are the roots of a polynomial of degree (N-1). The experimental signal is analyzed by fitting it to a sum of exponentials. However, even the simple mechanism above requires a fit to three exponentials and data are rarely accurate enough to fit more than two exponentials with confidence.

The reaction of ADP with subfragment-1 can be treated as a two-step reaction which is sufficiently complex to illustrate the method of determining rate constants.

$$M + D \overset{(1)}{\longleftrightarrow} M \cdot D \overset{(2)}{\longleftrightarrow} M \cdot D^*$$

The binding of ADP increases the tryptophan fluorescence emission by 7 to 10% in the $M \cdot D^*$ state. The three states M, $M \cdot D$, and $M \cdot D^*$ are denoted as X_1, X_2, and X_3. The rate equations are

$$\dot{X}_2 = k_1 [D] X_1 - (k_2 + k_{-1}) X_2 + k_{-2} X_3$$

$$\dot{X}_3 = k_2 X_2 - k_{-2} X_3$$

The equilibrium constant is $K = K_1(1 + K_2) \approx K_1 K_2$ for $K_2 >> 1$. The solution of the rate equations for the formation of $M \cdot D^*$, the only observable species, is

$$M \cdot D^*/Mo = (K[D]/(K[D] + 1)) \left(1 + \frac{\lambda_2}{\lambda_1 - \lambda_2} \exp(-\lambda_1 t) - \frac{\lambda_1}{\lambda_1 - \lambda_2} \exp(-\lambda_2 t)\right)$$

where λ_1 and λ_2 are the roots of the quadratic equation $\lambda^2 + b\lambda + c = 0$, in which $b = k_1[D] + k_{-1} + k_2 + k_{-2}$ and $c = k_1[D](k_2 + k_{-2}) + k_{-1}k_{-2}$. The exponential terms have opposite signs, consequently, the signal will show an initial lag corresponding to the build up of the $M \cdot D$ state.

At sufficiently high concentrations of D, $k_1[D]$ is the largest quantity in both b and c which become $b = k_1[D]$ and $c = k_1[D](k_2 + k_{-2})$. Since the two roots of the quadratic

are $-2\lambda_{1,2} = -b \pm b(1-4c/b^2)^{1/2}$ and $4c/b^2 << 1$, the square root can be expanded and approximated to yield $\lambda_1 = k_1[D]$ and $\lambda_2 = k_2 + k_{-2}$. Since $\lambda_1 >> \lambda_2$, the first exponential term decays very fast, a lag is not observable, and the time dependence reduces to $(1-\exp[-(k_2+k_{-2})t])$. Thus, measurements at high ligand concentration determine $k_2 + k_{-2}$.

At low ligand concentrations, the relation between the k_f and λ are more complex but two limiting cases are important. First, if $k_{-1} << k_2$ the roots are $k_1[D]$ and $k_2 + k_{-2}$. If $k_1[D] << k_2 + k_{-2}$ in the low range of ligand concentrations, the signal again fits a single exponential, $\lambda = k_1[D]$, and the rate increases linearly with [D]. Second, if $k_{-1} >> k_2 + k_{-2}$, the roots are $K_1(k_2 + k_{-2})[D]$ and $k_2 + k_{-2}$. At low concentrations, the rate is $\lambda = K_1(k_2 + k_{-2})[D]$. In either case, a plot of λ vs. [D] is linear in [D] at low concentrations and the slope determines the apparent second-order rate constant k^a. In case 1 it equals k_1 and in case 2, $k^a = K_1(k_2 + k_{-2})$.

In the intermediate range of concentrations in the first case, there should be a ligand concentration such that k_1D is comparable to $k_2 + k_{-2}$, the two roots being of the same magnitude and the signal showing a distinct lag. In the second case, the first step is essentially in equilibrium throughout the transient phase because the rate constant for reaching equilibrium is $k_1[D] + k_{-1} > k_2 + k_{-2}$ for all [D] and, thus, there is no lag. The square root can be expanded and the transient then fits a single exponential with

$$\lambda = \bar{K}(k_2 + k_{-2})[D]/(\bar{K}[D] + 1)$$

$$\bar{K} = \frac{k_1}{k_{-1} + k_2} \simeq K_1$$

In this case, the variation of rate with concentration fits a hyperbola.

The demonstration that the rate reaches a maximum at high concentrations is presumptive evidence that the signal is given by an isomerization of an intermediate state. The absence of a lag and the observation of a hyperbolic variation of λ with concentration indicates a two-step mechanism in which the initial complex is in rapid equilibrium with ligand. The binding of ADP satisfies these criteria with $k_2 + k_{-2} \simeq 100$ to 150 sec^{-1}, $k^a = 1.5 \times 10^6\ (M)^{-1}\ sec^{-1}$, and $K_1 \simeq 10^4\ M^{-1}$ (100 m*M* KCl, pH 7, 20°C). An example of an experimental record is shown in Figure 3 together with the fit to a single exponential term.

For a number of enzyme reactions, the initial binding of the ligand approaches the maximum rate set by diffusion which is approximately $10^8\ M^{-1}\ sec^{-1}$.[61] In the example, k^a is much smaller than the diffusion-limited rate and it is unlikely to be a pure rate constant. Thus, a low value of k^a is further support for a rapid equilibrium. From the value of K_1 and an assumed value for k_1 of $10^7 - 10^8\ M^{-1}\ sec^{-1}$, k_{-1} is $10^3 - 10^4\ sec^{-1}$, which satisfies the requirement of $k_{-1} >> k_2 + k_{-2}$. In the example, k_{-2} can be measured by displacing ADP with excess ATP or pyrophosphate and has a value of 2 sec^{-1}. Therefore, $K = K_1K_2$ which is $7.5 \times 10^5\ M^{-1}$ and in reasonable agreement with the association constant determined by equilibrium methods.

The rate constant k_1 is also affected by ionic strength if the ligand and the binding site contain charged groups. Ionic reactions between small molecules generally obey the relation $\log(k_1/k_1^0) = C\mu^{1/2}$ ($C = 1.02\ z_a z_b$, where z_a and z_b are units of charge of the reactants, k_1^0 is the rate constant at zero ionic strength and infinite dilution, and μ is the ionic strength).[16] A low value of k_1 relative to that expected from a diffusion-limited process, could occur at low ionic strength if the ligand and binding site have like charges. In this case, the rate would increase with ionic strength. In the ADP example, k^a decreases with increasing ionic strength and the low value cannot be explained by a charge effect. The finding that the rate of ADP binding reaches a maxi-

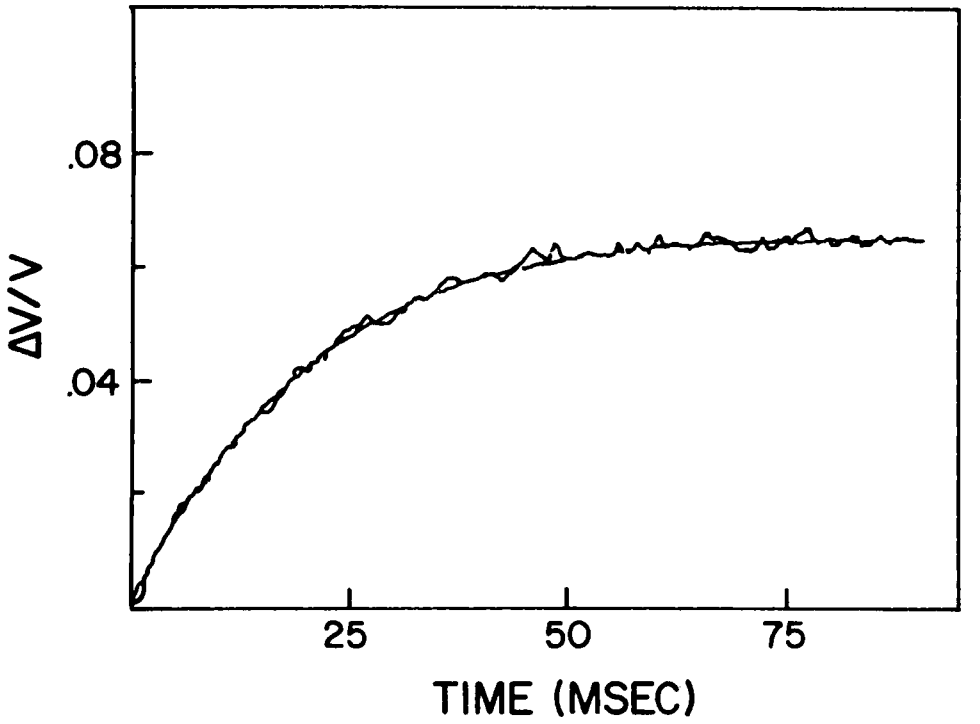
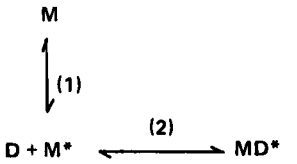


FIGURE 3. An example of a stop-flow record, the enhancement of protein tryptophan fluorescence for the binding of ADP to SF-1. (25 μM ADP, 3 μM SF-1, 100 mM KCl, 10 mM $MgCl_2$, pH 7, 20°C). The irregular line is the experimental signal, the smooth line is the curve obtained by the least squares fit to a single exponential term. Apparent rate constant $\lambda = 47.6\ sec^{-1}$.

mum value is not conclusive evidence for the mechanism since the protein may exist in two or more conformations, only one of which binds the ligand.



If $K_1 = M^*/M < 1$, the apparent rate constant of association at low ligand concentrations is K_1k_2 and the maximum rate at high ligand concentrations is $k_1 + k_{-1}$. The kinetic behavior is identical with the previous mechanism. Therefore, auxiliary evidence is needed to rule out this alternative. For example, different ligands which bind to the same site would give the same maximum rate because this parameter is a property of the protein. If both states bind ligand, the concentration dependence of the rate is complex and could be distinguished in a favorable case.

If the rate increases linearly with ligand concentration over the experimental range, the quantity measured is k^* and it may be difficult to decide whether the quantity is a pure rate constant k_1 or a mixed constant of the form K_1k_2, the only criteria being the magnitude of k^* and the presence or absence of a lag. This is also the case in measuring the rate of association of myosin or myosin intermediates with actin because very high concentrations of proteins are not accessible. There is very little evidence on rate constants of protein-protein association reactions, but one would expect the rate constant for the formation of the initial complex to approach the diffusion limit. A 10 to 100

times smaller diffusion constant for proteins vs. small molecules would suggest second-order rate constants of 10^6 to 10^7 M^{-1} sec^{-1}. Values in this range have been obtained for the association of SF-1 with actin.[241] The rate constant also obeyed the ionic strength relation, although the slope of a plot of log k_1 vs. $\mu^{1/2}$ may be a poor estimate of the product of charges at the binding site as the equation was derived for spherical ions.

The ADP association reaction illustrates a common problem in kinetic analysis. The actual mechanism may contain more than two steps but a two-step model is sufficient to account for the published data at pH 8 and 20°C. At lower pH and lower temperature the fluorescence signal is clearly biphasic; consequently, there are at least two transitions giving rise to a change in fluorescence emission.[101] It is important to examine a reaction over a wide range of pH conditions, ionic strength, and temperature before deciding that a particular mechanism is satisfactory.

Examples of Kinetic Schemes

In discussing the kinetic schemes, one needs the analysis of some of the simpler mechanisms:

1. The four-state mechanism presents a complex general solution because the signal must be fitted to three exponential terms. A particular case of the following scheme probably describes the transient evidence for SF-1.

$$M + T \underset{}{\overset{(1)}{\rightleftharpoons}} MT_1 \overset{(2)}{\rightleftharpoons} MT_2 \overset{(3)}{\rightleftharpoons} MD \overset{(4)}{\rightleftharpoons} M + D$$

The first step is assumed to be a rapid equilibrium, the second to be essentially irreversible ($k_{-2} << k_3$), and the fourth step to be slow compared to the third ($k_4 << k_3 + k_{-3}$). The first two steps can then be represented as a single process of rate $\lambda = K_1k_2[T]/(K_1[T] + 1)$ and writing $\bar{k}_3 = k_3 + k_{-3}$, the solution for a saturating substrate concentration is

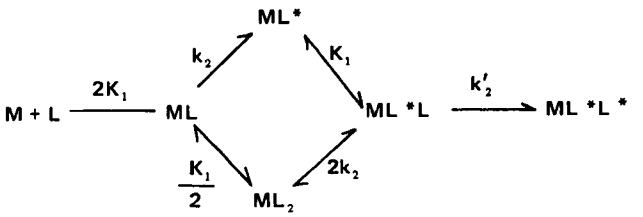
$$\frac{M \cdot D}{Mo} = (K_3/(1 + K_3))\left(1 + \frac{\bar{k}_3}{(\lambda - \bar{k}_3)} \exp(-\lambda t) - \frac{\lambda}{(\lambda - \bar{k}_3)} \exp(-\bar{k}_3 t)\right)$$

$$\frac{M \cdot T_2}{Mo} = 1/(1 + K_3)\left(1 + \frac{(\bar{k}_3 - \lambda(K_3 + 1)}{(\lambda - \bar{K}_3)}\right) \exp(-\lambda t) + \frac{K_3 \lambda}{(\lambda - \bar{k}_3)} \exp(-\bar{k}_3 t)) \quad (2)$$

2. Positive cooperativity is a mechanism in which the binding of ligand or an isomerization step increases the binding constant or isomerization rate for the second ligand. It has been considered to be a possible mechanism for myosin or actomyosin reactions. For example, consider the simple case of a rapid equilibrium and a single isomerization. For a single site (subfragment-1),

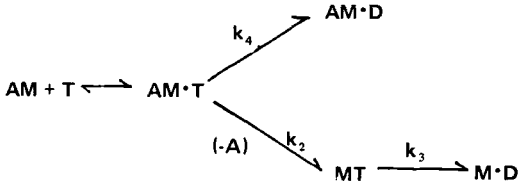
$$M + L \overset{K_1}{\rightleftharpoons} ML \overset{k_2}{\rightleftharpoons} ML^*$$

the rate is $\lambda = K_1k_2[L]/(K_1[L] + 1)$. For two binding sites which are identical for reaction with a single ligand, the scheme is



for the case in which the first isomerization increases the rate of isomerization for the second ligand ($k_2' > k_2$). The solution depends on two rate constants — $\lambda_1 = 2k_2K_1[L]/(K_1[L]+1)$ and $\lambda_2 = k_2'K_1[L]/(K_1[L]+1)$. The fluorescence signal F is proportional to ML* + ML*L + 2ML*L* and is given by the expression, $F = 2 - (\lambda_1/(\lambda_1-\lambda_2)) \exp(-\lambda_2 t) + ((2\lambda_2-\lambda_1)/(\lambda_1-\lambda_2)) \exp(-\lambda_1 t)$. In the limit of strong cooperativity, $k_2' >> 2k_2$ and $\lambda_2 >> \lambda_1$ and the signal is $F = 2(1 - \exp(-\lambda_1 t))$. Since λ_1 is twice the value for a molecule with a single binding site, the rate constant for myosin is twice the rate constant for subfragment-1. Similar conclusions hold if the association constant K_1 is increased or if both K_1 and k_2 are increased by the binding of the first ligand. A similar problem arises in the dissociation of actomyosin by ATP.

3. The branching pathway mechanism is seen in dissociation vs. hydrolysis in the acto-SF-1 mechanism. At high substrate and low actin concentrations, the reversal of dissociation can be neglected.



The experimental result is that k_2 is much greater than the rate of hydrolysis which requires $k_2 >> k_3 + k_4$. The maximum measured rate of hydrolysis of the first ATP is $k_3(1 + k_4/k_2) \simeq k_3$. The transient rate of hydrolysis for acto-SF-1 divided by the rate for SF-1 alone is $1 + k_4/k_2$.

One will also need steady-state solutions for actomyosin ATPase. As these are obtained by standard methods, the equations are presented as needed.

Chemical Properties of Myosin

An important question for the interpretation of the enzyme mechanism is whether myosin is a dimer. Myosins from various types of muscle always consist of six polypeptide chains — two of 200 to 205 kD called heavy chains (HC) and four light chains (LC) of weights from 16 to 27 kD. The molecular weights of the light chains are somewhat variable for the different muscle types and the terminology is even more so. However, it is now reasonably clear that there are two types of light chains. The first has a molecular weight of 16 to 20 kD, a Ca (Mg) binding site and is phosphorylatable by a specific protein kinase (LC kinase). The light chain can be removed without any major effect on the hydrolytic step or steady-state ATPase activity of myosin and is responsible for Ca regulation in myosin-linked systems (type 2). It is released by treatment with the thiol agent, 5,5′-dithio-bis(2-nitro)benzoic acid (DTNB), at low divalent metal concentrations in the case of fast striated muscle. The homologous light chain is removed by EDTA treatment in molluscan muscle. Thus, it has been termed the DTNB light chain, the EDTA light chain, or the LC-2 light chain in cardiac muscle. Sequence analogies have been found for light chains of various muscles with troponin-C, the Ca binding portion of troponin and with parvalbumin, a Ca binding protein present in many muscle types.[109] The interchange of light chains between molluscan and vertebrate white, cardiac, and smooth muscle myosins with at least partial restoration of Ca control[104] emphasizes the similarity of this light chain in different types of myosins. It is reasonable to use a common terminology and, following the suggestion of S. V. Perry, this first light chain will be referred to as the P-light chain (P-LC).

The second type of light chain is difficult to remove and once removed invariably leads to a loss of ATPase activity. It is referred to here as the A-light chain. In the case of red skeletal muscle, the myosin appears to have two different light chains with a slightly different weight or mobility.[177,234] In rabbit skeletal myosin, two A-light chains are present with molecular weights of 20.7 and 16.5 kD. Sequence studies[52] show these polypeptide chains to be the same except for an additional 4-kD piece. In this case and any case in which two chains can be distinguished, the larger is referred to as A1 and the smaller as A2.

There is increasing evidence that the composition of a myosin molecule is either $(HC)_2$ $(P\text{-}LC)_2$ $(A1)_2$ or $(HC)_2$ $(P\text{-}LC)_2$ $(A2)_2$. The ratio of A1 to A2 light chains in a myosin preparation is 1.2 to 0.8.[115] The antibodies to the extra peptide of A1 react only with one half of the myosin population.[80] A preliminary report indicates partial separation of HMM into two fractions enriched in either A1 or A2.[223] At this level of resolution, myosin is a dimer and two myosin isozymes are present in myosin preparations even when the protein is obtained from a single muscle fiber.[235] As discussed in the next section, the properties of SF-1 containing either light chain (SF-1, A1 and SF-1, A2) are very similar. Microheterogeneity has been detected in the heavy polypeptide chain.[202] Thus, myosin is not a perfect dimer and there is no preference for the association of A1 and A2 with the two types of heavy chains.[170]

A final statement requires the complete amino acid sequence, but the chemical evidence provides no support for a mechanism in which intrinsic differences in kinetic properties are ascribed to the two heads of a particular myosin molecule. The problem of cooperativity is discussed below. The enzymatic properties of SF-1 will be considered first on the assumption that the simplest system is the place to begin.

Properties of SF-1 ATPase

Earlier studies established some important properties which apply to SF-1 ATPase, although they were often made on myosin or HMM. The steady-state rate of SF-1 ATPase is very low, 0.05 to 0.06 sec^{-1} (100 m*M* KCl, 20°C), yet approximately one ATP is rapidly hydrolyzed in the initial phase of the reaction. This phenomena, first described by Weber and Hasselbach,[232] is termed the early phosphate burst and was extensively studied by Tonomura and colleagues who established the basic steps in the mechanism.[102] The kinetic scheme proposed by Tonomura is relatively complex because different kinetic properties are ascribed to the two myosin heads, thus, forming two kinds of SF-1 molecules. The contributions of Tonomura have not been properly recognized. However, because of the complexity of the complete kinetic scheme, the evidence will not be presented here but in a separate section.

Lymn and Taylor[121] made an estimate of the rate of the hydrolysis step and also showed that the products of hydrolysis dissociate slowly approximately at the steady-state rate.[210] The minimum kinetic scheme is

$$M + ATP \underset{}{\overset{(1)}{\longleftrightarrow}} M \cdot ATP \overset{(2)}{\longleftrightarrow} M \cdot Pr \overset{(3)}{\longleftrightarrow} M + ADP + P_i$$

In it, k_2 is approximately 140 sec^{-1} or larger (50 m*M* KCl, pH 8, 20°), k_3 is the steady-state rate, and k^+_1 is 10^6 M^{-1} sec^{-1}.

This first effort had several shortcomings. In particular, it was suggested that M·Pr might simply be a complex of ADP and P_i with myosin but several experiments showed that M·Pr was distinguishable from M·ADP or M·ADP·P_i (difference spectrum,[148] spin resonance spectrum,[185] and fluorescence enhancement[237]). A major step in understanding the mechanism was made by Bagshaw and Trentham and collaborators[8,11,220] by stop-flow fluorescence measurements and the use of substrate analogues. A seven-

step mechanism was proposed which became widely accepted and will serve as a starting point for the discussion of the current evidence. Mechanism 1 is

$$M + ATP \overset{(1)}{\rightleftharpoons} M \cdot ATP \overset{(2)}{\rightleftharpoons} M \cdot ATP^* \overset{(3)}{\rightleftharpoons}$$

$$M \cdot Pr^{**} \overset{(4)}{\rightleftharpoons} M \cdot ADP \cdot P_i^* \overset{(5)}{\underset{(Pi)}{\rightleftharpoons}} M \cdot ADP^* \overset{(6)}{\rightleftharpoons}$$

$$M \cdot ADP \overset{(7)}{\rightleftharpoons} M + ADP$$

where M · Pr** refers to a product intermediate state.

A similar scheme omitting step 5 was suggested by Koretz and Taylor[108] based on transient proton release and phosphate measurements. There is general agreement that the rate-limiting step occurs after hydrolysis and is considerably slower than the preceding steps. Thus, the mechanism can be divided into two parts, the series of steps up to hydrolysis (step 3) and the steps in product release, which considerably simplifies the analysis.

Asterisks denote states of enhanced tryptophan fluorescence relative to SF-1; although the symbols are not intended to be quantitative, the fluorescence of M · Pr** may be roughly twice M · ATP*. The maximum fluorescence enhancement for SF-1 prepared by papain digestion is approximately 20%.[237] The fluorescence signal was fitted to a single exponential term (100 m(*M*) KCl, pH 8, 20°). The apparent rate λ reached a maximum value at high ATP concentrations, and the dependence on concentration was fitted to a hyperbola which defined an apparent association constant of 5×10^3 M^{-1} and a maximum rate of 400 sec^{-1}. No lag was observed at any ATP concentration, and the first-order rate constant at low ATP concentrations was 1 to 2×10^6 M^{-1} sec^{-1}, which is in reasonable agreement with the value obtained from phosphate measurements by Lymn and Taylor.[121] These results satisfy the criteria for a two-step rapid equilibrium mechanism, and the maximum rate of the fluorescence signal was assigned to step 2 ($k_2 = 400$ sec^{-1}).

As discussed in the section on kinetics, a four state mechanism should be fitted by three exponential terms but the absence of a lag suggests that the initial step is a rapid equilibrium which reduces the time dependence to two exponentials. Since a single term fitted the data, there are a number of possible explanations. The problem is avoided if the fluorescence signal occurs only at step 2. This explanation was rejected because other ligands, such as AMPPNP (which is not hydrolized) or ATP-γS (which does not give a rapid hydrolysis step), produce only a 10% enhancement. The enhancement with ATP of 20% could be explained if M · ATP* gives a 10% change and the hydrolysis step adds a further 10% increase. The behavior of ADP strengthens this argument since, as already discussed, the reaction satisfies a two-step rapid equilibrium mechanism and the rate constant for the fluorescence transition was found to be the same as that for ATP. However, the amplitude was only 6% for papain SF-1.

To explain why the fluorescence change associated with the hydrolysis step is not observed as a separate signal, it was suggested that k_3 is much larger than k_2; consequently, the apparent rate of hydrolysis is determined by k_2. The phosphate measurements[121] were made only up to a concentration of 2×10^{-4} (*M*) in ATP, which is below the concentration necessary to saturate the rate. In a further study,[108] it was noted that the maximum rate of the hydrolysis step could not be calculated from the data in this concentration range and that the Bagshaw-Trentham interpretation is consistent with this evidence.

The magnitude of the phosphate burst reported by Lymn and Taylor[121] was 0.7 to 0.8 mol per site, although a somewhat lower value had been obtained by Kanazawa

and Tonomura.[102] A plausible explanation for a value less than one was proposed by Bagshaw and Trentham.[10] A tenfold excess of SF-1 over ATP-γ^{32}P gave roughly a 90% hydrolysis in 0.2 sec. Dilution with excess unlabeled ATP had no effect on the hydrolysis of the remaining 10%, which proceeded at the steady-state rate. This experiment showed that the substrate is tightly bound in the M·ATP* state ($k_{-2}<0.02$ sec^{-1}). The less than stoichiometric hydrolysis at the end of the transient phase can be explained if K_3 is relatively small. A burst of 0.9 would correspond to $K_3 = 10$. Since $k_3 >> k_4$, M·ATP* and MPr** would essentially be in equilibrium in the steady state and the burst would be $MPr^{**}/Mo = K_3/(K_3+1)$ (Equation 2, $t \rightarrow \infty$). A study of the formation of enzyme bound ATP from ADP and P_i allows K_2 to be calculated from the known value of K_1, as determined by Bagshaw and Trentham. A value of 10^7 to 10^8 was obtained which established that the substrate binding is essentially irreversible.[123,124,243]

The scheme for the first three steps is seen to be a reasonable interpretation of the evidence, although the assignment of the rate constants of fluorescence transitions is based on indirect arguments. Also, at pH 8 and 20°C the reaction is very fast and experimental errors prevent an adequate test of the fit of the concentration dependence of the rate to a hyperbola.

The fluorescence and phosphate signals have been reinvestigated for a range of conditions of ionic strength, pH, and temperature.[31,193,196,209] The maximum rate of the fluorescence signal decreases markedly with temperature (E_a = 100 kJ/mol degree) and also decreases with decreasing pH. At pH 7 and 3° (50 m*M* KCl), the fluorescence rate is 10 to 15 sec^{-1} as compared to 125±20 sec^{-1} at 20°C. At 3°C, the rate of hydrolysis is roughly 10 sec^{-1} but, again, the maximum was not determined accurately.[209] However, quenching with excess unlabeled ATP during the transient phase showed that further hydrolysis occurred in 1 to 2 sec following the quench. If $k_3 >> k_2$ and K_3 is relatively small, then M·ATP* and M·Pr** must be essentially in equilibrium during the transient phase and no increase in hydrolysis would have been expected.

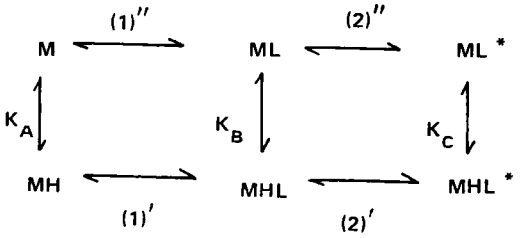
Further studies[30,31,99] require some revisions of the interpretation of the fluorescence steps. The lower rate at pH 7 and 20°C permits a more accurate analysis of the kinetics. The signal shows a small deviation from a single exponential when analyzed by computer fitting procedures, but the deviation is not sufficiently large to justify the assigning of more than one rate constant. The concentration dependence of the rate deviates markedly from a hyperbola. (This same result was obtained by Chock and Eisenberg.)[30] The discrepancy is very clear in experiments in which SF-1 has been separated into the two species, SF-1, A1 and SF-1, A2. The SF-1 made by the chymotryptic digestion procedure of Weeds and Taylor[236] gave a fluorescence enhancement of 36 to 38% as compared to 22 to 25% for papain SF-1 measured in a fluorimeter in the presence of excess ATP or in the stop-flow apparatus at low ATP concentrations. The difference probably results from the loss of the P-light chain in the chymotryptic digestion method. In addition, the amplitude of the observed fluorescence signal of chymotryptic SF-1 decreases to 20 to 23% with increasing ATP concentration in stop-flow experiments after correction for dead time, yet the final amplitude at the end of the transient is the same. Calibration of the stop-flow signals verified that the loss of signal arises from a very fast fluorescence change which is completed within the dead time of the apparatus (1.4 msec). There is a smaller loss in signal amplitude at low temperatures or high ionic strength.

A tentative explanation for these results is the occurrence of two fluorescence transitions, as was originally postulated by Bagshaw and Trentham, except that k_2 is very fast ($\sim$ 1000 sec^{-1}) and k_3 is the maximum observed rate at high ATP concentrations. The amplitudes of the two steps are 10 to 12% and 24 to 26%, respectively, for chymotryptic SF-1 based on the loss of signal. This interpretation is supported by measurements of hydrolysis at low ionic strength (10 m(*M*) KCl, pH 7, 10°C).[30,99] A max-

imum rate is reached at less than 100-μM ATP of 25 sec^{-1}, which is approximately equal to the rate of the fluorescence step. Quenching of labeled ATP binding by mixing with excess unlabeled ATP showed the tight binding of ATP to be complete in 10 msec or less which is ten times faster than the observed fluorescence signal. Thus, recent studies are consistent with the mechanism proposed by Bagshaw and Trentham but with the important difference that the rate of the observed fluorescence signal at high ATP concentrations probably measures the rate of the hydrolysis step.

A fraction of a proton is released in the transient phase which provides another method of measuring the rate constants. The amplitude of the signal is 0.2 to 0.3 mol/mol of sites at pH 8. (0.3, myosin[51] 0.23, SF-1;[11] 0.4, HMM;[29] 0.25, SF-1, myosin;[108] and 0.2, SF-1[191]). The amplitude decreases as pH is reduced from 8 to 6.5,[191] while there is only a small change in the size of the phosphate burst.[209] Thus, the proton is neither stoichiometric nor equal to the size of the phosphate burst and is the same magnitude for AMPPNP, α, β-methylene ATP, and ADP, which are either not hydrolyzed or do not give a phosphate burst.[11,108,129,191] The proton must be derived from a change in the pK of one or more protein groups and could occur in steps 1, 2, or 3. If the contribution from steps 1 and 2 is the same for hydrolyzed and nonhydrolyzed ligands, little or no proton release occurs in step 3.

Consider a two-step mechanism with one ionizable group (ADP binding):



The observed constants K_1 and $k_2 + k_{-2}$ are pH dependent. If the ionization step is fast compared to k_2, which is probably the case, the transitions between M and MH, ML and MHL, etc. can be treated as equilibria. The molecular constants k'_2 and k_2'' are then related to k_2 by $k_2 = k_2' + \alpha(k_2''-k_2')$, where α is the degree of ionization of MHL, $\log \alpha/(1-\alpha) = pH - pK_B$. Therefore, k_2 will vary with pH according to a titration curve with pK corresponding to pK_B. If K_1 or K_2 depend on pH, then $K_A \neq K_B$ or $K_B \neq K_C$ and a fractional release or absorption of a proton must occur.

The proton release observed in stop-flow experiments at high ligand concentrations such that k_2 is concentration independent must be derived from step 2. If a single group undergoes a shift in pK, the maximum proton release Q occurs at $pH = (pK_B + pK_C)/2$ and $\Delta pK/2 = \log (1-Q)/(1+Q)$. Kinetic and pH meter measurements gave a maximum burst at pH 8.3 to 8.5, but the fit to a single group was poor.[191] Marsh et al.[129] fitted the data obtained in pH meter measurements to two groups, pK 8.45 and 9.4 which shift to pK 8.0 and 9.1, respectively. Below pH 7, the burst increased and was approximately fitted by the shift of a group from a pK of 6.6 to 6.3. To date, the kinetic data are not sufficiently accurate to separate the contributions from the steps in the reaction scheme.

The rate of proton release with ATP as substrate was the first signal used in kinetic analysis of the transient phase.[51] In the original studies with myosin, a poor fit was obtained to a single exponential term. Later studies with HMM in 0.5 M KCl[29] and SF-1 and myosin at various ionic strengths[108] gave satisfactory fits to a single term. At 25° C, Chock and Eisenberg found a linear increase in rate with ATP concentration, while an approach to a plateau was obtained in the Koretz-Taylor measurements at 20°C. The rate was essentially equal to the rate of phosphate formation for low to moderate ATP concentrations.

The proton measurements emphasize that there is something wrong with the interpretation of the kinetic schemes of ATP vs. ADP. Equal magnitudes of proton release for ATP, ADP, and AMPPNP have been taken to mean no proton release in hydrolysis (step 3). Proton release in steps 1 and 2 of the ATPase scheme would require the proton rate to be a measure of k_2 (which is much faster than the observed fluorescence signal); however, this conclusion is contrary to the available evidence. It is inappropriate to discuss preliminary data here except to note that the problem may be with the ADP reaction. The association of ADP (and also AMPPNP) is probably a three-step reaction, and at least a part of the proton signal may come from the third step in both the ATP and ADP reactions.

The quenching studies of Bagshaw and Trentham provided evidence that K_3 is relatively small and, consequently, the phosphate burst should be less than unity. A phosphate burst of 0.85 ± 0.05 per site for SF-1, HMM, and myosin (pH 8, 20°C) was reported by Taylor,[209] a value of 0.9 for SF-1, A1 and SF-1, A2 by Taylor and Weeds,[207] and 0.8 for HMM by Seidel.[184] In the Seidel study, the burst was similar in magnitude for CTP, UTP, GTP, ITP, and tripolyphosphate. However, Tonomura and colleagues have obtained values of 0.5[92] and Yazawa et al.[247] have obtained 0.65 per site for HMM. The reason for this discrepancy is not known.

At low temperatures and pH 7, the burst was reduced to 0.5 ± 0.1.[30,209] The low value could be explained by a decrease in K_3 with temperature and possibly a slight decrease with decreasing pH. A pH-temperature jump applied at the end of the transient phase should lead to ATP hydrolysis or synthesis, depending on the direction of the jump. This prediction was qualitatively confirmed, although the extent of synthesis was smaller than expected from the values of K_3 at the two levels of pH and temperature.[209]

The low burst size might also be explained by a difference between myosin heads with only one giving a fast hydrolysis step at low temperature. However, measurements of intermediate oxygen exchange provide evidence for a low value of K_3 and do not depend on measuring the stoichiometry of the reaction. Exchange is expected to occur by the reversal of the hydrolysis step.

$$M \cdot ATP^{*} \underset{-H_2O,\ k_{-3}}{\overset{+H_2O,\ k_3}{\rightleftharpoons}} M \cdot ADP \cdot P_i^{**}$$

One oxygen is incorporated into phosphate in the cleavage step. Further incorporation can occur if the decay of the $M \cdot ADP \cdot P_i^{**}$ state into free products is slow compared to the rate of reversal (k_{-3}) and if the phosphate group can rotate to allow a different oxygen atom to be released in the H_2O. Conversely, if the substrate is ATP in which the three oxygens of the terminal phosphate are labeled with ^{18}O, reversal of the reaction would then lead to loss of ^{18}O from the enzyme-bound ATP. The rate of ^{18}O exchange determined by either method is a measure of k_{-3} or the rate of rotation of the phosphate group whichever is slower.

It is difficult to evaluate the ^{18}O exchange studies since there are discrepancies in the evidence presented by various laboratories and the results obtained by new techniques are incomplete. Rapid oxygen exchange occurs with SF-1 ATPase and appears to involve all four phosphate oxygens[12,190,194] The values of k_{-3} at 20 and 0°C are approximately 10 to 20 sec^{-1} and 1 sec^{-1}, respectively. These numbers combined with kinetic measurements of k_3 give values of the equilibrium constant K_3 which are about twice as large as the values obtained from phosphate burst measurements, but considering the errors involved the agreement is reasonable.

The results with myosin are less clear. Earlier work[221] and a recent study[189,190] indicated that only three quarters of the oxygens can exchange rapidly. Shukla and Levy proposed that restriction of rotation of the phosphate group could prevent the ex-

change of the fourth oxygen. They also showed that the extent of rapid exchange obtained with myosin HMM and SF-1 depended on the method of preparation, consequently the effects of proteolytic digestion as well as the loss of the P-light chain might account for the different rates and extent of exchange shown by myosin and its subfragments.

The method of analysis does not distinguish between a single reaction in which a maximum of three ^{18}O atoms are incorporated in phosphate and an average value of three ^{18}O per phosphate arising from two different reactions, one giving complete exchange and the other giving the incorporation of a single ^{18}O in the cleavage step. The problem can be resolved by determining the distribution of species containing zero to four ^{18}O atoms per phosphate by mass spectral analysis of the phosphate or by ^{31}P NMR spectroscopy. Results of Sleep et al.[194] and Webb et al.[231] support rapid exchange of all four oxygens in the myosin ATPase reaction which raises the problem that myosin preparations may contain varying amounts of a second ATPase as an impurity.

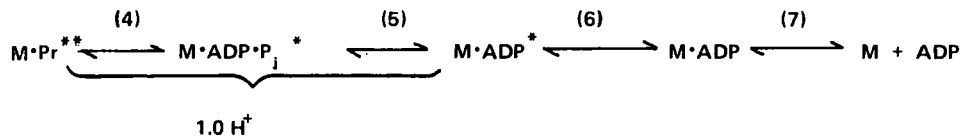
The technique of ^{18}O exchange is a powerful method which is capable of resolving a number of questions concerning the mechanism of ATP hydrolysis. At least for SF-1 the method provides the clearest evidence for reversal of the hydrolysis step and for a low value of the equilibrium constant K_3.

It has been proposed[250] that step 3 may not involve an actual cleavage of ATP, but rather a pseudo-rotation producing a strained apical bond between the γ phosphate and ADP moieties. The phosphate burst would then arise from hydrolysis occurring when the protein is denatured. The nature of the complex produced in step 3 is not known and it may be prudent to retain the symbol $M \cdot Pr^{**}$ rather than $M \cdot ADP \cdot P_i^{**}$ used by Bagshaw and Trentham. One piece of evidence which could support pseudo-rotation is the absence of proton release in step 3 at pH 8 since a free phosphate would ionize at this pH. Alternately, the environment of the bound phosphate would be required to shift its pK to above 9. The pseudo-rotation mechanism requires that the strained ATP bond be broken upon denaturation of the protein in acid, base of 6*M*guanidine hydrochloride. A distinction between pseudo-rotation and hydrolysis can be made by ^{18}O exchange measurements since in the pseudo-rotation mechanism the β-γ bridging oxygen should not exchange with the β-phosphoryl oxygens in ATP (for a fuller discussion of this question see Trentham et al.[221]). Preliminary evidence from this type of experiment indicates that the pseudo-rotation mechanism is incorrect.[219]

Product Release Steps

The rate of release of reaction products has been measured by rapid chromatography,[196,210] decay of the fluorescence signal in a single turnover of ATP,[11] release of the proton produced in ATP hydrolysis at pH 8,[11,29,196] and an increase in conductance.[196] The dissociation of ADP has been measured by blocking the rebinding of ADP with ATP, ATP analogues, or pyrophosphate.[11,196,220] The rate of dissociation of products is essentially equal to the steady-state ATPase rate at 20°C and is much slower than the dissociation of ADP.

The evidence is consistent with a simple sequential mechanism proposed by Bagshaw and Trentham.



The binding of ADP by a two-step mechanism has been discussed. The fluorescence enhancement of $M \cdot ADP^*$ and $M \cdot Pr^{**}$ are approximately 7% and 20%, respectively (papain SF-1). In 100 m*M* KCl at pH 8 and 20°, $k_4 \sim 0.05$ sec^{-1} and $k_6 = 2.5$ sec^{-1}.

The rate of P_i release from $M \cdot P_i$ or the complex formed from M, ADP, and P_i is very fast compared to k_4 or k_6, and the dissociation constant of P_i is approximately 1 m*M*. The binding of P_i to $M \cdot ADP$ or $M \cdot ADP^*$ is weaker than to M itself,[11] which indicates some preference for an ordered dissociation mechanism. The nature of the state $M \cdot ADP \cdot P_i^*$ is uncertain and labeling it with a single asterisk implies the same fluorescence enhancement as $M \cdot ADP^*$, while there is no evidence for this assignment. If this state is the same as the state obtained by adding a high concentration of phosphate to $M \cdot ADP^*$, the rate of phosphate dissociation must be very fast, and in the hydrolysis mechanism, this state would be converted to $M \cdot ADP^*$ as fast as it is formed. Consequently, it is not observable in transient experiments. The apparent rate constants of fluorescence decay, proton release, and increase in conductance as a measure of P_i release are equal,[196] and the order in which these changes occur cannot be decided, i.e., the pathway

$$M \cdot Pr^{**} \xrightarrow[P_i \downarrow H^+]{(4)} M \cdot ADP^{**} \xrightarrow{\text{fast}} M \cdot ADP^*$$

would lead to the same observable results. Studies on the reversal of hydrolysis[124] appeared to provide evidence for the $M \cdot ADP \cdot P_i^*$ intermediate and gave a value for K_5, but later studies[54] have not confirmed the earlier work. Steps 4 and 5 could not be separated; however, the equilibrium constant for the combined steps from MPr** to $M \cdot ADP^*$ was 0.19 (*M*) at pH 8. The omission of step 5 simplifies discussion without altering any observable property. Step 4 appears to be a slow conformational change which allows fast release of phosphate and a proton. Whether the H^+ comes from the release of $H_2PO_4^-$ which ionizes immediately or from a protein group is not decidable. The product release steps follow an ordered pathway, but it has not been proven that the order arises from steric considerations. The magnitude of k_4 depends on the divalent metal associated with the enzyme product complex. The rate increases in the order of Mg<Mn<Ca<no divalent cation. It is also increased by modification of the SH-1 sulfhydryl group.[196]

The rate of ADP dissociation decreases markedly with temperature and it was suggested that step 4 could be rate limiting at low temperature.[136] The point was established, though somewhat indirectly, by Bagshaw and Trentham[11] by calculating k_6 from the amplitude of the fluorescence at low temperature. The ratio of k_6/k_4 was 2 at 5°C. It was also shown that the amplitude at the end of the transient with ATP in excess decreased again before a true steady state was reached. This is evidence for a sequential pathway and for the presence of $M \cdot ADP^*$ in the steady state. That is, initially MPr** is formed and is in equilibrium with $M \cdot ATP^*$; this stage is followed by an increase in $M \cdot ADP^*$ at the expense of $M \cdot ATP^*$ and MPr** and, consequently, a decrease in fluorescence at rate $k_4 + k_6$ in the approach to the true steady state.

Direct measurements of k_6 as a function of temperature employing pyrophosphate displacement yielded similar results,[196] although k_6/k_4 was 3 at 3°C. Thus, ADP dissociation would become slower than k_4 at a temperature of −5 to −8°C. The steady-state rate for the Bagshaw-Trentham scheme at saturating substrate concentration is

$$V = \left(\frac{K_3}{K_3 + 1}\right) \frac{k_4 k_6}{k_4 + k_6}$$

An Arrhenius plot of the steady-state rate would be expected to show curvature if there is a switch in the rate-limiting step at low temperature because the slope is determined by the activation of energy of k_4 at high temperatures and k_6 at low temperatures. The Arrhenius plot for myosin-Mg ATPase is generally reported to be linear, but Watterson et al.[230] reported some curvature. The SH-1 modified enzyme exhibits a nonlinear

Arrhenius plot[113] and a transition at 13°C has been reported for Manganese ATPase.[82] Kinetic measurements of k_4 and k_6 gave a crossover temperature of about 5°C for SH-1 modified enzyme and at 11 to 13°C for Mn ATPase. The Arrhenius plot calculated from k_4 and k_6 was noticeably curved in the latter case in agreement with steady-state rate measurements, but the sharp transition was not observed. The rate-limiting step for Mn ATPase at low temperature is ADP release.[6,82,196,247]

Summary

Kinetic studies on SF-1 ATPase are adequately described by the Bagshaw-Trentham mechanism.

$$M + ATP \underset{}{\overset{K_1}{\rightleftharpoons}} M \cdot ATP \overset{k_2}{\rightleftharpoons} M \cdot ATP^* \overset{k_3}{\rightleftharpoons} MPr^{**} \overset{k_4}{\rightleftharpoons} \underset{H^+ P_i}{\downarrow}$$

$$M \cdot ADP^* \overset{k_5}{\longrightarrow} M \cdot ADP \overset{K_6}{\rightleftharpoons} M + ADP$$

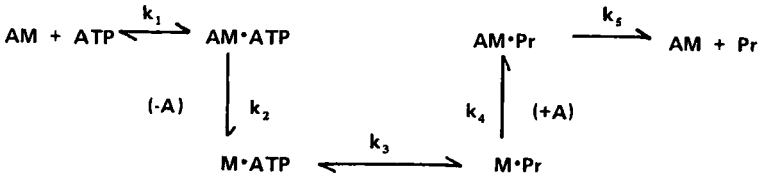
The $M \cdot ADP \cdot P_i^*$ state is omitted for simplicity since its inclusion has no observable consequence in discussing the SF-1 and acto-SF-1 mechanisms. There are two transitions in which tryptophan fluorescence emission is enhanced (steps 2 and 3). The maximum rate of the observable fluorescence transition is probably k_3, which is equal to the rate of hydrolysis. The apparent second-order rate constant obtained at low ATP concentrations is $k^a = K_1k_2$. As more accurate measurements did not confirm a hyperbolic dependence of rate on ATP concentration, the apparent association constant reported by Bagshaw et al.[8] and Koretz and Taylor[108] does not measure K_1. The rate constant k_2 for the first fluorescence transition is larger than k_3 but is not accurately known (100 m*M* KCl, pH 7, $\sim$1000 sec^{-1}, 20°C). Substrate binding is essentially irreversible because k_2 is extremely large (10^7 to 10^8); k_3 depends on pH and in 100 m*M* KCl at pH 7 it is 125 ± 25 sec^{-1} at 20°. The complexes $M \cdot ATP^*$ and MPr^{**} are in equilibrium in the steady state, $K_3 \sim 6$ to 10. The rate-limiting step at 20°C is k_4 (0.05 sec^{-1}) and it appears to be a conformation change leading to the fast release of P_i. $M \cdot ADP^*$ is an intermediate state of the hydrolysis pathway equivalent to the state formed by adding ADP to SF-1. At low temperature, k_4 and k_5 are similar in magnitude, while for modified SF-1 or Mn ATPase at low temperature, $k_5 < k_4$ and ADP dissociation is the rate-limiting step.

It should be noted that recent studies which indicate that the fluorescence signal measures k_3 rather than k_2, as originally proposed by Bagshaw-Trentham, require further substantiation, and this interpretation is not acceptable without a corresponding revision in the mechanism of binding of nucleotides (ADP, AMPPNP).

Acto-SF-1 ATPase

A key to the understanding of the actomyosin ATPase mechanism was provided by Eisenberg and Moos.[49] The activity of acto-SF-1 in excess of that of SF-1 increased with actin concentration according to a hyperbolic relation, i.e., $V = V_M[A]/(K_M + [A])$. The maximum ATPase activity V_M obtained by extrapolation to infinite actin concentration was 200 times larger than SF-1 ATPase ($V_M \sim 10$ sec^{-1}, pH 8, 20°, low ionic strength.) V_M did not appear to be very dependent on ionic strength while K_M increased markedly with increasing ionic strength. (Throughout the discussion, K_M refers to the concentration dependence of actin activation at saturating substrate concentrations.) In fact, in 100 m(*M*) KCl which is taken to be roughly physiological, K_M was very large ($\simeq 10^{-3}$ M). Since it is difficult to exceed 100 μM in actin concentration, the V vs. [A] plot showed very little curvature and the value of V_M was subject to large errors.

Kinetic studies of dissociation of acto-SF-1 and the rate of hydrolysis in 100 or 50 m*M* KCl at 20°C showed that the rate of dissociation increased in proportion to ATP concentration, exceeding 500 to 1000 sec^{-1}, while the rate of hydrolysis appeared to reach a maximum of about 150 sec^{-1} and was not distinguishable from the rate for SF-1 alone.[122] The following scheme was proposed:

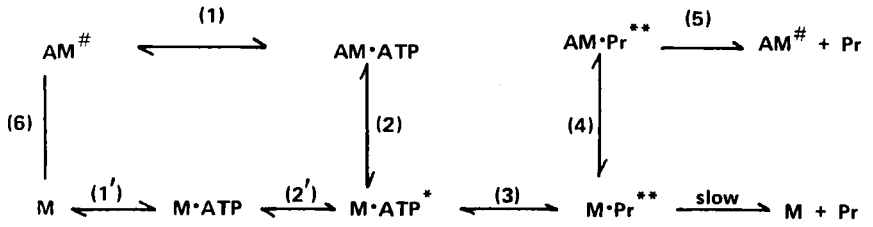


The hydrolysis step occurs on free SF-1 and actin recombines with the M·Pr complex. This mechanism appeared to be consistent with the Eisenberg-Moos studies of the steady state. The slowest first-order step should determine the maximum rate and $V_M << k_3$. The equation for the steady-state rate as a function of actin concentration is easily obtained if reversal of all steps is ignored. At a high ATP concentration, $k_1[ATP] >> k_3$, $k_2 >> k_3$, $V = V_M[A]/(K_M + [A])$, $V_M = k_5$, and $K_M = k_5/k_4$.

An interesting feature of this mechanism is the dissociation and recombination of the actomyosin complex for each turn of the ATPase cycle. Since structural models require association and dissociation of the cross bridge in the mechanochemical cycle, the biochemical scheme fits in a natural way to the structural model. This was one of the main reasons for adopting the biochemical scheme — the original evidence was based on measurements of dissociation and hydrolysis, while the rate of recombination was not properly measured. An estimate of the recombination rate was given by Koretz et al.[107]

Subsequent studies have provided further evidence in support of the main points of the mechanism. At 3°C, the rates of the hydrolysis step and the fluorescence signal are much slower than at 20°C (100 m*M* KCl, pH 7, 15 to 20 sec^{-1}), while the rate of dissociation remains greater than 750 sec^{-1}. Chock et al.[31] and Sleep and Taylor[193] found the rate of the fluorescence signal at high ATP concentrations to be essentially the same for acto-SF-1 and SF-1. The rate of the hydrolysis step reached a plateau of 10 sec^{-1} for acto-SF-1 and roughly the same value for SF-1.[193] Phosphate measurements are subject to relatively large errors, but within a factor of 2 the fluorescence and phosphate rates were equal for acto-SF-1 and SF-1, although the fluorescence rate was slightly larger than the phosphate rate in each case. These studies established that dissociation occurs before hydrolysis since the rate constants of the two processes differ by at least a factor of 50.

Proper measurements of the rate of formation of a complex between M·Pr and actin were made by the double mixing technique.[241] Slightly less than one ATP was mixed with SF-1 and after 2 sec, which allowed the system to form the M·Pr state, the system was mixed with actin. This method is superior to using a regenerating system[107] or calculating the rate constant from a single turnover experiment (one ATP mixed with acto-SF-1 produces dissociation followed by recombination.[31,81]) For a range of actin concentrations in which the excess ATPase activity is proportional to actin concentration, the rate of recombination was equal to the turnover rate of acto—SF-1 ATPase, as required by the scheme. This result was documented for a range of temperatures and ionic strengths and provides additional support for dissociation-recombination as the major pathway. The scheme can be expanded to include the states defined by fluorescence enhancement. Mechanism 2 is



A serious disagreement exists in the studies of Chock et al. vs. Sleep and Taylor regarding the amplitude of the fluorescence signal which occurs after dissociation. The former reported a fluorescence after dissociation of about 10% using papain SF-1 which was associated with step 3. If the total signal is 20% and step 2′ is assumed to give a 10% enhancement, the evidence fits satisfactorily with the Bagshaw-Trentham scheme. But, it also leads to a contradiction with the scheme as originally formulated since the maximum rate of the fluorescence signal was assigned to k_2' and k_3 was assumed to be much larger than k_2'. On this basis, one would expect the rate of the fluorescence signal for acto-SF-1 to be much larger than for SF-1. The discrepancy is not a temperature effect since the rates are also equal at 20°.[100] Sleep and Taylor worked largely with chymotryptic SF-1 and, basing measurements on signals at high ATP concentrations, found a 20 to 23% fluorescence signal after dissociation which was equal to the signal for SF-1 alone. It was assumed that the observed fluorescence represented the total signal for SF-1. Quenching with cold ATP showed the substrate to be irreversibly bound in the first dissociated state prior to the fluorescence transition. Based on the assignment of the fluorescence transitions in the Bagshaw-Trentham scheme, it was concluded that the first dissociated state could not be part of the myosin mechanism and it was given the symbol M·ATP*. The problem has probably been resolved by the reinvestigation of the myosin scheme by Johnson and Taylor and by Chock and Eisenberg as described in the previous section.

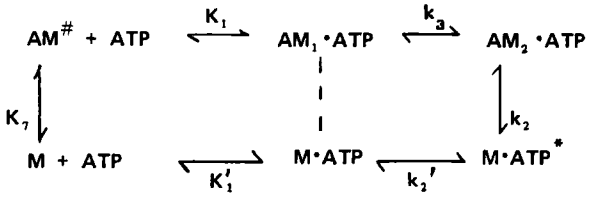
The amplitude of the observed fluorescence signal for chymotryptic SF-1 decreases with increasing ATP concentrations to a value of 23%, but the amplitude of the acto-SF-1 signal is 23% independent of ATP concentration. This discrepancy is resolved when one recognizes that the rate measured by fluorescence at high ATP concentrations is k_3 rather than k_2' and the amplitude of this signal is 23% after correcting for the dead time. The missing 10 to 12% of fluorescence change for acto-SF-1 is approximately accounted for by an increase in fluorescence in the formation of the acto-SF-1 complex. It is not certain that the step A + M ↔ AM# accounts for all of the signal because it is necessary to correct for scattering and there could be a small discrepancy. The symbol AM# is used for the associated state rather than AM*, since the conformation of myosin need not be the same as in the M·ATP* state.

The steps in dissociation have not been specified for rabbit SF-1. Its rate of dissociation is proportional to ATP concentration which defines k_1', but k_2 is not defined because it is too fast to measure. By analogy with ATP binding to myosin, it can be asked whether the encounter complex is in rapid equilibrium with substrate and if a conformation change precedes dissociation. In the case of SF-1 from slower muscles, a maximum dissociation rate has been measured (Eccleston et al.,[44] arterial slow muscle; and Marston and Taylor,[135] anterior lattissimus dorsi [ALD], gizzard [smooth], and cardiac muscles of the chicken). The ALD and smooth muscles give maximum rates of dissociation of about 500 sec^{-1}, 10 m*M* KCl, pH 7, 3°) and a hyperbolic dependence of rate on ATP concentration. At the least these experiments prove that an AM·ATP state exists and has a value of 2×10^3 M^{-1} for the apparent association constant, one of roughly the same magnitude as the apparent association constant for the initial ATP binding to SF-1.

Thus, the minimum mechanism of dissociation is AM# + ATP $\overset{K_1}{\rightleftharpoons}$ AM·ATP $\overset{k_2}{\rightleftharpoons}$ A + M·ATP*. A consideration of the rate of ATP dissociation provides indirect evi-

dence for a second actomyosin-ATP state. Measurements of the rate of ATP synthesis gave values for ATP dissociation of greater than 0.05 sec^{-1} [243] and 0.2% of the rate of hydrolysis.[164] The rate of displacement of ATP from the M·ATP* by actin has been measured both from the release of radioactive ATP and from intermediate oxygen exchange by Sleep et al.[195] This experiment measures the relative rate constants of the reactions $M \cdot ATP^* + A \xrightleftharpoons{k^a_{-2}} AM + ATP$ and $M \cdot Pr^{**} + A \xrightleftharpoons{k^a_4} AM + Pr$; $k^a_{-2} \simeq (1/3)k^a_4$. Although the rate constant of ATP release can only be given relative to a particular kinetic scheme, the experiment establishes the important result that dissociation of actomyosin is readily reversible. The rate constant k^a_4 has been measured,[241] and based on the reversible dissociation mechanism discussed later in this section, the effective rate constant of ATP dissociation from actomyosin is the order of 50 sec^{-1} (10 m(*M*) KCl, 23°C). The minimum estimate of the rate of ATP dissociation shows little dependence on the kinetic scheme, and the rate is 10^5 times larger than the rate of dissociation of ATP from the M·ATP* state.

The rate is surprisingly large; however, it is small compared to the rate of dissociation expected for a rapid equilibrium of the AM·ATP complex with free ATP. A plausible scheme is



where $K_1 \simeq K_1' \simeq 10^3(M)^{-1}$, $K_7 \simeq 10^7\ M^{-1}$, $k_2' > 1000\ sec^{-1}$, and $K_2 \simeq 10^7$ to 10^8 (100 m(*M*) KCl, 20°C). The maximum rate of dissociation is k_a or k_2, whichever is smaller. The initial complex with ATP in both pathways is a collision intermediate that is in rapid equilibrium with ATP in the medium. The demonstration that dissociation is reversible requires M·ATP* to be essentially in equilibrium with actin. Therefore the displacement experiment of Sleep et al. measures the quantity K_2k_{-a} where $K_2 = k_{-2}/k_2$. The rate of dissociation is greater than 1000 sec^{-1}; consequently, k_a is the same magnitude as k_2' or possibly somewhat larger. The equilibrium constant K_a of actomyosin is reduced relative to K_2' approximately in the ratio of the rates of dissociation of ATP from actomyosin and myosin, namely by a factor of 10^5, $K_a \sim 10^2$ to 10^3. From the "thermodynamic box" argument, K_2 is 10^2 to $10^3 M^{-1}$. The mechanism is completely described by assigning the rate and equilbrium constants, and the interaction between actin and myosin or ATP and myosin in terniary complexes cannot be split up into individual terms. However, differences in equilibrium constants of similar transitions (K_7 and K_2 or K_a and K_2') may be thought of as defining an interaction free energy.[69] In this sense, the very tight binding of ATP to myosin provides the energy to drive a transition to a state in which actin is weakly bound to myosin ($AM_2 \cdot ATP$).

The association reactions of actin with M, M·ATP*, and M·Pr** are expected to exhibit similar rate behavior although with different magnitudes of the equilibrium constants; $A + M \xrightleftharpoons{K_7} AM$, $A + M \cdot ATP^* \xrightleftharpoons{K_2} AM_2 \cdot ATP$, $A + M \cdot Pr^{**} \xrightleftharpoons{K_4} AM \cdot Pr.^{**}$ The rate constant k_7 is $10^7\ (M)^{-1}\ sec^{-1}$ at low ionic strength, which is close to the limit set by diffusion,[241] while k_{-7} is 0.2 sec^{-1}.[130] The apparent rate constant k^a_4 is almost 100 times smaller at the same ionic strength and decreases with ionic strength; consequently, the low value cannot be attributed to charge repulsion. In the case of slow muscles, the values are 10 to 50 times smaller than for rabbit psoas SF-1, while k_7 is unchanged. These results indicate that the measured quantity is not a pure rate constant, and since it is proportional to the turnover rate of the actomyosin ATPase, it is simply explained by a rapid equilibrium mechanism $A + M \cdot Pr^{**} \xrightleftharpoons{K_4}$

$AM \cdot Pr^{**} \overset{k_5}{\rightarrow} AM + Pr$. The actual formation of the $AM \cdot Pr^{**}$ is a fast reaction, but the association constant K_4 is small, and the complex is stabilized by the release of products. The rate is $\lambda = K_4 k_5[A]/(K_4[A] + 1)$, and the parameter measured at low actin concentrations is k_4^a (equal to $K_4 k_5$). If we suppose that k_5 is roughly the maximum rate of the actomyosin ATPase (see the discussion of steady-state models to follow), the value is of the order of 10 sec^{-1}. Thus, K_4 is 10^4 to 10^5 M^{-1} at low ionic strength and decreases with increasing ionic strength.[241] For a simple collision (as appears to be the case for the association with myosin in the absence of nucleotides), k_4 would be of the order of 10^7 M^{-1} sec^{-1}, and consequently, k_{-4} is 10^2 to 10^3 sec^{-1}. In the corresponding reaction of $M \cdot ATP^*$ with actin, the dissociation constant k_2 is of the order of 1000 sec^{-1}, k_2^a is similar to k_4^a,[195] and as already discussed, K_2 is 10^2 to 10^3 M^{-1}.

The evidence indicates that the association-dissociation steps in the ATPase cycle are fast reactions which may be close to equilibrium in the steady state. A different explanation for the slow rate of complex formation between actin and $M \cdot Pr^{**}$ has been proposed and will be considered next.

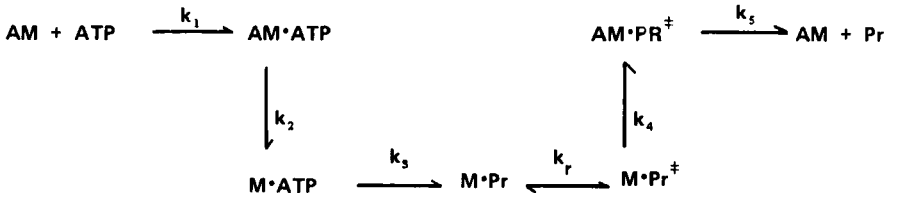
The Refractory State

The kinetic scheme (mechanism 2) is a satisfactory model for the behavior in moderately dilute solutions. Eisenberg and colleagues asked the important question: Is this mechanism correct at high actin concentrations which approach the effective concentration in muscle? In the earlier studies,[122] values were assigned to apparent rate constants in the forward direction. If the reversal of all steps is neglected, the scheme predicts $V = V_M[A]/([A] + K_M)$. For convenience, all quantities are normalized by dividing by the myosin concentration Mo. The degree of association $\theta = (AM \cdot ATP + AM \cdot Pr) \simeq AMPr$ at high ATP concentrations because $k_1^a(ATP)$ and k_2 are very much greater than k_3 and k_5. At 20°, $k_3 >> V_M$ and, consequently, $V_M = k_5$. Since $AM \cdot Pr = [A]/([A] + K_M)$, then $V/\theta = V_M = k_5$. Thus, the model predicts that the rate and degree of association have the same dependence on actin concentration and, thus, at 90% of V_M, the system should be 90% associated. It should be noted that V_M and K_M are experimental quantities defined by a double reciprocal or other suitable plot. The expressions for V_M and K_M in terms of rate constants are properties of the particular model. Since the reversal of all steps was neglected, K_M must be a ratio of rate constants, $K_M = k_5/k_4$.

Measurements of association made in the scanning ultracentrifuge were compared with V/V_M, and it was clearly shown that the predictions of the scheme are incorrect.[45,48] At an actin concentration such that V is nearly equal to V_M, the degree of association was only 0.4 for acto-HMM and 0.2 for acto-SF-1. Even lower values were obtained using viscosity and light scattering, but these assays are nonlinear.

The low association even at high actin concentrations is explainable if there is a state in the myosin scheme which does not combine with actin. The model contained the states inferred from transient studies, but it is always possible that a "silent" transition with appropriate kinetic properties has escaped detection. The noncombining intermediate was termed the "refractory state."

Further evidence for the refractory state was obtained by measuring the rate of reassociation of acto-SF-1 in single turnover experiments as a function of actin concentration.[31] The rate obtained by fitting the later stages of recombination to a single exponential term gave a hyperbolic dependence on actin concentration which paralleled the concentration dependence of the steady-state rate. Thus, the apparent rate of recombination of a myosin product intermediate determines the ATPase activity at all actin concentrations rather than only in the linear range. The results were interpreted from the following model, mechanism 3:



The extra step is the transition from the refractory state M·Pr to a nonrefractory state M·Pr‡. If we treat this scheme as an essentially irreversible mechanism (only the reversal of the refractory transition is included) and assume $k_r << k_5$, the apparent rate of recombination in a single turnover is approximately

$$k^a = \frac{k_r [A]}{[A] + K_M}$$

$$V = \frac{k_r [A]}{[A] + K_M}$$

and

$$\theta = \frac{(k_r / k_5) [A]}{[A] + K_M}$$

where $K_M = (k_r + k_{-r})/k_4$. The modified model accounts for the stated observations. The concentration dependence of k^a and V is the same, $V_M = k_r$, and the fraction association at V_M is k_r/k_5 (the expressions are essentially those of Chock et al.).[31] Furthermore, $V/\theta = k_5$ and from the experimental results for acto-SF-1, $V_M = 0.9$ sec^{-1} and $\theta = 0.2$; the calculated value of k_5 is 4.5 sec^{-1} (no added KCl) and pH 7, 5°).

An alternative method of determining a maximum rate of acto-SF-1 ATPase is to measure the rate at a constant actin concentration and extrapolate to infinite SF-1.[48] The maximum rate obtained by this method is 5 to ten times larger than the maximum obtained by extrapolating to infinite actin concentration. The two quantities obtained by these extrapolations are referred to as V_M (SF-1→∞) and V_M (A→∞). This result is expected from the refractory state model because the refractory step is by-passed by excess M·PR‡ which combines with actin and $V_M = k_5$. In these experiments, the value of V_M per actin residue depended also on actin concentration. However, this problem cannot be explained by the simple model and will be discussed later.

The refractory state model is attractive because it explains the low association at the apparent maximum rate of hydrolysis, the different values of V_M obtained by the two methods of extrapolation, and correctly predicts the value of k_5 from the measurement of V/θ. However, the conclusions are based on an essentially irreversible kinetic scheme which is probably not applicable to concentrated actin solutions. The hydrolysis step is reversible, and at low temperature and ionic strength, the phosphate burst is approximately 0.5 mol per site, which indicates that K_3 is approximately 1. As discussed in the last section, the dissociation-reassociation steps are probably sufficiently fast for these steps to be close to equilibrium in the steady state. Extrapolation to infinite actin is meaningless unless the kinetic scheme includes the reversal of all steps in which actin is a reactant. Furthermore, the reversal of the dissociation step indicates that there could be an appreciable fraction of AM·ATP present at high actin concentrations; therefore, it is necessary to consider the effect of direct hydrolysis of ATP without dissociation.

If the predictions of the irreversible scheme of Lymn and Taylor[122] are compared with the essentially irreversible refractory state scheme of Eisenberg,[31] the latter scheme

is clearly superior; however, neither is satisfactory at high actin concentrations. The steady-state behavior of plausible kinetic schemes is considered in the next section.

Steady-state Models

Steady-state rate equations for various kinetic schemes are summarized in Table 1. To simplify the discussion, the equations refer to a saturating substrate concentration. In this case, the release of products can be treated as an irreversible step (k_5) which determines the rate of binding of ATP. The minimum kinetic scheme obtained from transient measurements has four intermediate states which are conveniently referred to as A·MT, A·MD, MT, and MD; MT corresponds to M·ATP*, and MD to M·Pr**.

Models *I* and *II* are the schemes implied by the earlier studies of Lymn and Taylor[122] and Eisenberg,[48] respectively, in which dissociation by ATP and reassociation of MD are treated as irreversible steps. Reversal of the hydrolysis step is included, and no assumption is made concerning the magnitude of k_5/k_3. The refractory state was introduced to explain the low association at the apparent V_M observed at low ionic strength and temperature. Recent transient measurements for these conditions[30,100,209] gave the following results: a rate of hydrolysis of 8 to 10 sec^{-1} and a phosphate burst of 0.5, which yields $K_3 = 1$, $k_3 \sim 5$ sec^{-1}. Steady-state measurements gave V_M ($A \to \infty$) $\simeq 1$ sec^{-1}, V_M (SF-1$\to \infty$) $\simeq 5$ sec^{-1}. For either model, the rate at infinite SF-1 is k_5. Model *I* predicts V_M ($A \to \infty$) $= 2.5$ sec^{-1} and $\theta(V_M) = 0.5$. Thus, a low association at V_M is expected from the relative magnitudes of the rate constants; however, the degree of association and the value of V_M obtained experimentally is lower than predicted by model *I*.

A lower association at V_M can be obtained by introducing a refractory state, but the rate constant for the refractory state transition (k_r) is not equal to V_M. It enters the model as a free parameter which is adjusted to obtain agreement with the experiments. Since $\theta(V_M) = 0.2$[48] and $\theta(V_M) = V_M/k_5$, we have $k_5 = 5$ sec^{-1}, in approximate agreement with the value obtained by extrapolation to infinite SF-1 as already discussed. The value of k_r calculated from the expression for V_M is 3.3 sec^{-1}. k_r was also obtained from a single turnover experiment,[31] but this procedure would also underestimate k_r because it is comparable to k_3. Although model *II* is an improvement over model *I*, it does not provide strong evidence for an extra state, since agreement with experiment depends on fitting to an extra parameter. Model *II* also predicts the same concentration dependence of V and θ, which appears to be contrary to the experimental results.

Models *I* and *II* probably do not apply at high actin concentrations, since reversal of the dissociation and recombination steps has been omitted. A reasonable approximation is provided by treating steps 2 and 4 as rapid equilibria. For example, a perturbation of the equilibrium concentrations of A·MT and MT is restored at rate $(k_2 + k_{-2}[A]) > 1000$ sec^{-1}, which is ten times larger than the first order rate constants k_3, k_{-3}, and k_5.

Including the reversal of steps 2 and 4 leads to models *III* and *IV*. As was first pointed out by Lymn,[116,117] an extrapolation to infinite actin is meaningless if the reversal of step 2 is omitted, since the rate of this step becomes indefinitely large. The kinetic scheme implies that actin is an inhibitor of the ATPase at high actin concentrations, because the concentration of A·MT increases. If the rate of direct hydrolysis is zero, then at infinite actin, A·MT = 1 and the hydrolysis rate is zero. There is no evidence that k_d is identically zero; consequently, models *III* and *IV* refer to a system in which $k_d << k_3$. The steady-state rate will pass through an apparent maximum value at some value of A and decrease to the value k_d (or zero) at infinite actin. It is convenient to introduce the reduced concentration $\bar{A} = K_4A$ and the parameter $\alpha = K_2/K_4$, where K_2 is the association constant of MT with actin.

TABLE 1
Steady-state Kinetic Schemes for Acto SF-1

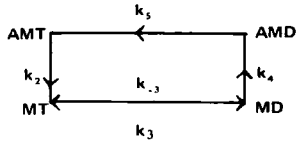
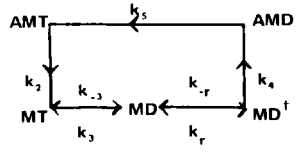
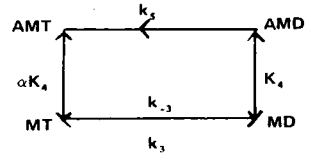
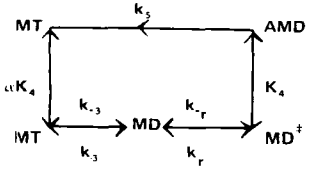
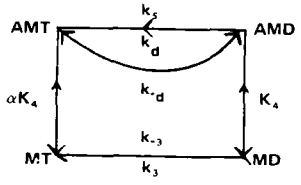
Model	Concentration dependence of V	V_M ($A \to \infty$)	K_M	$\Theta(V_M)$ Association at apparent V_M
I. Irreversible dissociation 	$V = \frac{V_M A}{A + K_M}$	$\frac{k_5}{1 + \frac{k_5}{k_3}}$	$\left(\frac{k_5}{k_4}\right)\left(\frac{K_3 + 1}{K_3}\right)\frac{1}{1 + \frac{k_5}{k_3}}$	$\frac{1}{1 + \frac{k_5}{k_3}}$
II. Irreversible dissociation refractory state 	$V = \frac{V_M A}{A + K_M}$	$\frac{k_r}{1 + \frac{k_r}{k_5}\left(1 + \frac{k_5}{k_3}\right) + \frac{1}{K_3}}$	$\frac{k_r + k_{-r} + k_{-r}/K_3}{k_4\left[1 + \frac{k_r}{k_5}\left(1 + \frac{k_5}{k_3}\right) + \frac{1}{K_3}\right]}$	$\frac{k_r/k_5}{1 + \frac{k_r}{k_5}\left(1 + \frac{k_5}{k_3}\right) + \frac{1}{K_3}}$
III. Reversible dissociation 	$V = \frac{k_5 \bar{A}/C}{A + K_M + \frac{k_5 \alpha \bar{A}^2}{k_3 C}}$ $C = 1 + \frac{k_5}{k_3} + \frac{\alpha}{K_3}$	$V = 0$, $A \to \infty$ Apparent maximum at $\bar{A} = \left[\frac{(K_3 + 1)k_3}{K_3 k_5 \alpha}\right]^{1/2}$	$\frac{K_3 + 1}{K_3 C}$	< 1

TABLE 1 (Continued)
Steady-state Kinetic Schemes for Acto SF-1

IV. Reversible, refractory 	$V = \dfrac{k_5 \bar{A}/C}{\bar{A} + K_M + \left(\dfrac{k_5\alpha}{k_3} + \dfrac{k_5}{k_r} + \dfrac{\alpha}{K_3}\right)\dfrac{\bar{A}^2}{C}}$ $C = 1 + \dfrac{k_5}{k_3} + \dfrac{k_5}{k_r} + \dfrac{1}{K_3}\left(\dfrac{k_5}{k_r} + \dfrac{\alpha}{K_r}\right)$	$V = 0,\ A \to \infty$ apparent maximum at $\bar{A} = \left[\dfrac{K_3 + 1 + K_r K_3}{\alpha k_5 K_3 K_r \left(\dfrac{1}{k_r K_3} + \dfrac{1}{k_3}\right)}\right]^{1/2}$	$\dfrac{K_3 + 1/K_r + K_3/K_r}{K_3 C}$	< 1
V. Reversible, direct hydrolysis 	$V = \dfrac{k_5 \bar{A}(1 + k_d \alpha \bar{A}/k_3)/C}{\bar{A} + K_M + \alpha\left(\dfrac{k_5 + k_d + k_{-d}}{k_3}\right)\dfrac{\bar{A}^2}{C}}$ $C = 1 + \dfrac{k_5}{k_3} + \dfrac{\alpha}{K_3} + \dfrac{\alpha k_d + k_{-d}}{k_3}$	$\dfrac{k_5}{1 + \dfrac{k_5}{k_d} + \dfrac{1}{K_d}}$ but apparent maximum if $\dfrac{k_d}{k_3} < 1$	$\dfrac{K_3 + 1}{K_3 C}$	< 1 (1 if $k_d \geq k_3$)

Note: Kinetic schemes derived for the case of saturating substrate concentration; $\alpha = K_2/K_4$, $\bar{A} = K_4 A$; equations are given in pseudohyperbolic form, K_M corresponds to a Michaelis constant if $\bar{A}^2$ term is neglected. V_M $(A \to \infty)$ is maximum rate extrapolated to infinite actin for models I, II, and V (if $k_r/k_3 \geq 1$); it is the maximum rate at $\frac{dV}{dA} = 0$ for models III, IV, and V (if $k_r/k_3 < 1$); $\Theta_{(V_M)}$ is the degree of association at concentration of actin for which the rate is a maximum. V is the rate per SF-1 in presence of actin minus the rate in the absence of actin.

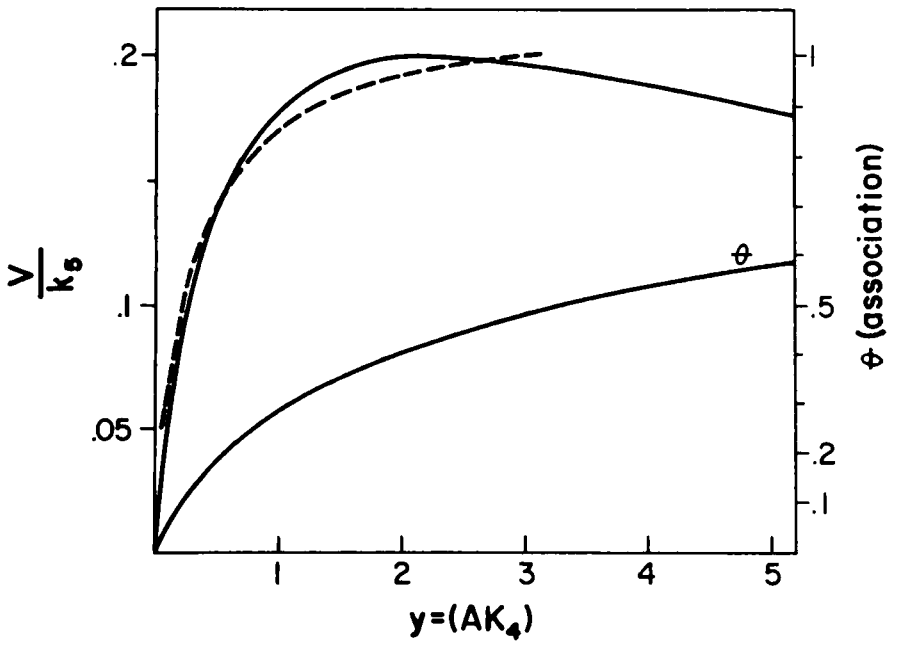


FIGURE 4. Normalized plot of the rate and degree of association for acto-SF-1 ATPase illustrating actin inhibition for the reversible dissociation model (model III, Table 1). Rate is expressed relative to k_5. The concentration variable is [A]K_4 (dimensionless) where [A] is actin concentration and K_4 is the association constant of M·Pr** with actin. The fractional association of the system is θ and the dashed curve is a hyperbola with a maximum value of 0.22. $K_3 = 1$, $k_3 = 5$ sec^{-1}, $k_5 = 10$ sec^{-1}, and $\alpha = 0.2$

The steady-state rate for model *III* reaches an apparent maximum value at an actin concentration $\bar{A}_M$, where $\bar{A}_M = [(K_3 + 1)k_3/(K_3k_5\alpha)]^{1/2}$. The behavior is best illustrated by a numerical example calculated for the same values of K_3 and k_3 used previously. It is easily shown for a rapid equilibrium model that V_M (SF-1 → ∞) = $k_5K_3/(K_3 + \alpha)$. Relatively weak inhibition by actin is assumed ($\alpha = 0.2$), and k_5 is taken to be 10 sec^{-1}, which corresponds to a measured value of V_M of 8.3 sec^{-1}. The concentration dependence of V and θ is shown in Figure 4. The dashed curve in the figure is a plot of a hyperbola with asymptote 0.22 to illustrate that the deviation of the data from a hyperbola will not be large unless the rate is within 20% of the apparent V_M. The apparent maximum rate is 2 sec^{-1}, and the degree of association at 90% of the maximum rate is 0.29.

A refractory state in a reversible scheme is a myosin intermediate state (MD) with a much lower association constant than MT or MD‡ (model *IV*). The general behavior is not altered by including an extra state, although the apparent maximum rate will be shifted to a higher actin concentration. The scheme has two extra parameters k_r and K_r with which to fit the experimental values of V_M and $\theta(V_M)$.

The models are incomplete, since the direct hydrolysis pathway A·MT $\underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}}$ A·MD $\xrightarrow{k_5}$ AM + D has been omitted. Reversal of dissociation was demonstrated by Sleep et al.;[195] consequently, A·MT is present at high actin concentrations, and although the rate constant k_d has not been measured in transient state experiments, it is necessary to consider the effect of direct hydrolysis on the steady-state scheme (model *V*). It is evident intuitively that the occurrence of an apparent maximum rate at some actin concentration in model *III* arises from the build-up of A·MT. Direct hydrolysis allows A·MT to be converted to A·MD, relieving the effect of actin inhibition, and because it provides a pathway which does not require dissociation, the system will show a higher association for a given actin concentration. The curve in Figure 4 was calculated

for $k_d = 0$. If k_d is allowed to increase while holding the other parameters constant, the apparent maximum rate will shift to higher actin concentrations; when k_d/k_3 is equal to one, the maximum occurs at infinite actin concentration, i.e., the concentration dependence of V is a hyperbola, $V_M(A \rightarrow \infty) = k_5k_d/(k_5 + k_d + k_{-d})$, and $\theta(V_M) = 1$. An important consideration is the relative contributions to the ATPase cycle by the dissociation-recombination pathway vs. the direct hydrolysis pathway. The ratio of the two fluxes for model *V* is $k_3/(k_d\alpha A)$.

It should be clear from the discussion that further evidence is necessary to determine the mechanism of acto SF-1 ATPase at high actin concentrations. The existence of an extra myosin intermediate state with low affinity for actin has neither been proven nor disproven. A low degree of association at the apparent V_M can be partly explained (without adding another state) simply from the relative magnitudes of the rate constants and an inhibitory effect of actin arising from reversal of the dissociation step. A better fit to the data can be obtained if a refractory state is included; however, this cannot settle the question, since the extra state provides an adjustable parameter. A different approach to the question is provided by intermediate-oxygen exchange studies by Sleep and Boyer.[192] The extent of intermediate exchange is determined by the lifetime of M·Pr**. In the original form of the refractory state model,[31] the limiting lifetime as actin approaches infinity is k_r^{-1}; therefore, appreciable intermediate exchange is expected. However, the experiments gave an extrapolated value of essentially zero. The reversible scheme without a refractory state predicts zero exchange or a small exchange if the direct hydrolysis pathway is included. At low temperature and ionic strength, the rate of hydrolysis k_3 is equal to or less than k_5, and M·ATP* satisfies the original definition of a refractory state.

The apparent maximum rate at high actin concentrations is not a well-defined parameter. Inhibition at a sufficiently high actin concentration is a necessary property of all models, although the concentration could be too high to be attained experimentally under some conditions. A decrease in rate at high actin concentrations has been observed at low ionic strength.[131] A proper test of a steady-state model requires the measurement of k_3, K_3, θ, and the dependence of the rate per site on actin and on SF-1 concentration. A proper test of a steady-state scheme has not been carried out.

Further evidence is also necessary to determine the contribution of the ATPase activity from the direct hydrolysis pathway. The problem can be resolved by comparing the concentration dependence of the rate per site obtained by varying the actin concentration (model *V*) with the dependence obtained by varying the SF-1 concentration. In the latter case, the rate equation for the reversible model is

$$V = \frac{k_5 M/(1 + \alpha / K_3)}{M + \left(\frac{K_3 + 1}{K_3 + \alpha}\right)\frac{1}{K_4}}$$

where V is the rate per G-actin and M is the SF-1 concentration. The equation is not affected by the magnitude of k_d, since the concentration of A·MD is determined by the three equilibrium constants αK_4, K_3, and K_4. It has been shown by Eisenberg and Kielley[48] that variation of the actin concentration gives a lower apparent V_M and a much lower concentration for the half-maximum rate than is obtained by varying SF-1. The differences arise from the accumulation of A·MT, which depends on the rate of direct hydrolysis. It is possible to determine all the constants from a combination of steady-state and transient measurements; however, the available evidence permits only a rough estimate. The ratio k_d/k_3 must be less than one to account for the very low association at the apparent V_M ($A \rightarrow \infty$) and for the differences in concentration dependence. The ratio could be small because the behavior is not sensitive to k_d/k_3 if the ratio is 0.1 or less. However, the available data are consistent with a value of 0.2.

Identity and Cooperativity of Myosin Heads

The chemical evidence indicates that myosin is essentially a dimer, but myosin preparations from rabbit white muscle consist of a mixture of at least two isozymes, each myosin molecule probably having either two A1 or two A2 light chains. There is general agreement that myosin has two binding sites for ATP, ADP, AMP PNP, and pyrophosphate, and competition experiments show that the various ligands are bound to the same site.[105,114,154,181,215,251] It must be determined whether the two sites are identical and independent.

Direct measurements of ligand binding constants by equilibrium dialysis have been interpreted as evidence for a single class of site for SF-1 or HMM,[114] for two classes,[179,215] and for positive cooperativity.[149] Binding has also been investigated by indirect methods in the sense that the signal amplitude is calibrated based on the maximum value at high ligand concentrations. Measurements of ADP binding by proton release,[129] fluorescence enhancement[196] and enthalpy change[106] fit a single class of site, one per head for SF-1 and HMM, while the ultraviolet difference spectrum has been interpreted as arising from only one head.[149]

The reactions with ATP have led to similar disagreements. Titration of the fluorescence or proton signal indicated a single class of site.[29,196] Phosphate burst measurements gave no indication of heterogeneity.[209] However, the amplitude of the burst as discussed in the previous section ranged from 0.5 to 0.9 mol per site in different laboratories. The steady-state ATPase activity did not fit a single Michaelis constant.[92,122,209] Some variation in association constants might be expected from the presence of two isozymes, yet the steady-state rate, phosphate burst, and fluorescence transients measured for SF-1, A1 and SF-1, A2 are hardly distinguishable.[99,207,236]

The question of cooperativity between sites is difficult to resolve. In globular proteins in which subunit interactions occur, the subunits are in contact and a mechanism can be postulated in which a conformation change induced in one subunit by ligand binding can be transmitted to other subunits. In the case of myosin, the heads appear to be in almost free rotation with respect to one another[139,212] and the presence of substrate does not affect the relaxation time. Cross-linking of heads has been obtained by d'Albis and Gratzer[36] which causes a significant change in sedimentation constant of the magnitude expected from the conversion from independent rotation to a fixed position. No change was noted in the steady-state ATPase activity. Since a much smaller change in sedimentation is detectable by differential methods and no change was found on adding substrate,[58] there is no evidence that the heads are in contact for an appreciable fraction of the time during the ATPase cycle. Thus, it is difficult to understand how a conformation change on one head could alter the affinity for substrate or the rate of the hydrolysis step of the second head. As discussed in the section on kinetics, the consequence of such a mechanism would be to increase the apparent rate of substrate binding by a factor of 2 for HMM relative to SF-1. In experiments in which HMM was converted to SF-1 by papain, no change in the tryptophan fluorescence rate was detected during the course of digestion within the experimental error of ±5%.[100]

To date, the questions of identity and cooperativity have not been settled. The problems arising from the heterogeneity or purity of myosin preparations and the effects of proteolytic cleavage of polypeptide chains in the preparation of HMM and SF-1 may possibly be responsible for the disagreements. The weight of evidence supports an identical independent site model in this reviewer's opinion, but this interpretation of the data would not be accepted by some investigators.

Acto-HMM ATPase

To understand the mechanism of HMM ATPase, one needs to understand why a system whose kinetic behavior should be complex seems surprisingly simple. The evi-

dence is described adequately by treating HMM as if it were two independent molecules of SF-1. The rate constants for dissociation by ATP, fluorescence transient, hydrolysis, association of HMM itself, or the product intermediate state with actin expressed per head are essentially equal to the corresponding values for SF-1 (Marston obtained a slightly higher value for the rate constant of association of HMM with actin).[51,130,193,241]

The steady-state ATPase satisfies the equation $V = V_M[A]/([A] + K_M)$. The maximum rate expressed per head is equal to the value for SF-1.[48,126,144,151] Eisenberg and collaborators and Moos reported K_M to be approximately one half of the SF-1 value. Margossian and Lowey gave values which were equal or slightly smaller. Eisenberg and collaborators have also shown that the degree of association at V_M is twice as large for HMM as for SF-1.

Answers are needed to the following questions about acto-HMM: Are both heads bound in the acto-HMM complex and in the intermediate complexes? How many molecules of ATP are required to dissociate acto-HMM? Are the heads independent or cooperative when attached to actin? What are the kinetic properties of mixed states such as HMM·ATP·Pr? It is often assumed that ATP must bind to both heads to produce dissociation. If this is the case, the sequential binding of two molecules of ATP would lead to a lag in the dissociation signal. By analogy with the acto-SF-1 reaction in which the rate constant of dissociation is proportional to ATP concentration, the simplest scheme is

$$AM + T \xrightarrow{2\,k_1 T} AM \cdot T \xrightarrow{k_1 T} AM \cdot T_2 \xrightarrow{\text{fast}}$$

A statistical factor is included for the two (presumably) identical heads. The time course of dissociation (D) for this mechanism is $D = 1-2\exp(-k_1(T)t) + \exp(-2k_1(T)t)$. This function shows a distinct lag which has not been detected. The discrepancy might be attributed to the use of light scattering to measure dissociation because the signal is slightly nonlinear[241] or to the AM·T state which because one head is detached, makes a smaller contribution to the light scattering. A second possibility is an oversimplification of the kinetic scheme since there is some reason to suppose that the initial binding of ATP is a rapid equilibrium followed by a conformational change or rapid dissociation. The solution for the corresponding rate equations is complex, but at least in the range in which the rate of dissociation is proportional to ATP concentration, the solution has the same form as the previous case with k_1 replaced by k_1^*, and again predicts a lag.

The lag would not be observed if the binding of a single ATP produced dissociation or if the binding was highly cooperative so that the effective rate of binding of the second ATP was much faster than the first. In either of these cases, the dissociation kinetics would follow the equation $D = 1-\exp(-2k_1^*t)$. (This problem is equivalent to the cooperative binding problem discussed in the last section.) Consequently, the rate of dissociation would be twice as fast for acto-HMM as compared to acto-SF-1 at the same ATP concentration, but this does not appear to be the case.

A similar problem occurs with the measurement of the apparent rate constant of association of the intermediate of HMM products with actin since the rate constant is approximately twice as large as for SF-1·Pr, which means that it may differ only by the statistical factor for two heads.[241] If the association is considered to be a rapid equilibrium, the attachment of the second head might be expected to influence the apparent rate constant.

Interaction between heads in the actin complex could occur since the heads are brought together by attachment to the adjacent G-actin residues. If both heads are bound independently to actin, what is the expected value of the association constant

compared to SF-1? Physical evidence indicates that both heads are simultaneously bound to actin because the loss of rotational freedom is a factor of 10^4 both for HMM and SF-1.[212] There is relatively poor agreement among measurements of the association constant of the acto SF1 complex. In 100 m*M* KCl, values range from 10^6 M^{-1} to 5×10^7 M^{-1}.[69,70,127,130] However, where a comparison has been made between acto-HMM and acto-SF-1 the association constant measured by the same technique is only 10 to 20 times larger for acto HMM.[70,72,127]

The independent binding of both heads might be expected to yield a very much larger association constant for acto-HMM. The problem of divalent ligand binding has been discussed by Jencks[98] for the case of small molecules. The magnitude of the equilibrium constant is larger than the square of the magnitude of the equilibrium constant of the corresponding monovalent ligand. However in the case of HMM, the equilibrium constant is expected to be much less than predicted for small molecules because of the almost complete rotational freedom of the two heads. The simplest two step binding scheme is

$$A + M \underset{}{\overset{2K_1}{\rightleftharpoons}} A \cdot M \overset{K_2}{\rightleftharpoons} A{:}M$$

where single and double dots refer to the number of attached heads and K_1 is the association constant for SF-1. The association constant K of HMM is given by $K = 2K_1(1 + K_2)$. If M_{eff} is the effective concentration of the second head in the vicinity of an actin site after the first head has attached then $K_1[M_{eff}] = [A{:}M]/[A \cdot M] = K_2$. A rough approximation to the value of M_{eff} is given by assuming the second head is distributed over the volume of a sphere of radius 100 to 150 A; consequently, M_{eff} is approximately $10^{-4}M$. A detailed treatment of the problem has been given by Crothers and Metzger[35] in the context of antibody binding. A value of K_1 of 10^6 to 10^7 M^{-1} leads to a value for K_2 of 10^2 to 10^3; thus, the association constant of HMM is expected to be 10^2 to 10^3 times larger than the value for SF-1. A further problem is the arrangement of the actin sites in an essentially linear array which will reduce the apparent affinity for higher degrees of occupancy of actin sites. The linear lattice problem for HMM binding has been treated approximately by Peller[165] and a more complete solution was given by Hill[74] for ligand binding to linear polymers. Inclusion of lattice effects does not alter the order of magnitude estimate of the association constant.

The experimental value of the ratio of the association constant of HMM and SF-1 is 10 to 100 times smaller than the value estimated for independent binding. Highsmith[72] has discussed the binding data in terms of negative cooperativity between the heads of HMM. The result can also be described by stating that the effective concentration of the second head is lower than expected for independent binding. Since the concentration of myosin heads in the muscle lattice probably exceeds $10^{-4}M$, the two heads of a particular myosin molecule could be attached to different thin filaments. Offer and Elliott[159] have proposed such a model based on electron microscope observations of individual myosin molecules and a consideration of the geometry of the muscle lattice.

The relatively small difference in association constants for SF-1 and HMM and the difficulties in interpreting the kinetics of dissociation-reassociation in the cycle suggest an interaction between heads in acto-HMM complexes. However, the behavior of the steady-state ATPase has been taken as evidence for independent activation of both heads by actin because V_M ($A \rightarrow \infty$) is twice as large for HMM as it is for SF-1.

Steady-state rate equations have been derived for acto-HMM for the same types of models considered in the discussion of acto-SF-1 ATPase. The equations are complex, and a detailed description is not warranted. Briefly, irreversible mechanisms lead to the wrong V_M or the wrong concentration dependence unless k_5/k_3 is very much greater than one. This condition does not appear to be satisfied at low temperature according

to the acto SF-1 data. Including possible cooperative or anticooperative steps does not improve the agreement. A reversible dissociation model has an extra free parameter compared to the acto-SF-1 case, since the equation for V_M depends on the relative association constants of MT_2, MTD, and MD_2 with actin. The parameters can be adjusted to give almost the same V_M per head as for acto SF-1 and a slightly higher association. However, if we can place confidence in the experimental result that the rate per head is equal for acto SF-1 and acto-HMM, then this equality should be deducible as a mathematic property of the model. Since we can make the rates only approximately equal by adjusting parameters, the models are judged to be unsatisfactory.

An obvious difficulty in the interpretation of the behavior of HMM is to discover the physical meaning of extrapolation to a high actin concentration. The steady-state rate equations assume the two heads are attached to the same actin filament, and the difficulty in reproducing the experimental results arises because association-dissociation steps for one head are influenced by the attachment of the other head. Yet the striking feature of the actin activation of HMM ATPase is that it behaves almost as if it were two independent SF-1 molecules. An obvious explanation of the same V_M per head with twice the degree of association is that the two heads interact with different actin filaments at high actin concentrations. At low ionic strength, the association of acto SF-1 is 0.2 at V_M. If the two heads of HMM bind independently to different actin filaments, the probability that at least one head is bound is 0.44. As long as the heads are undergoing the hydrolysis cycle independently, the V_M per head will be the same for any of the kinetic schemes we have discussed.

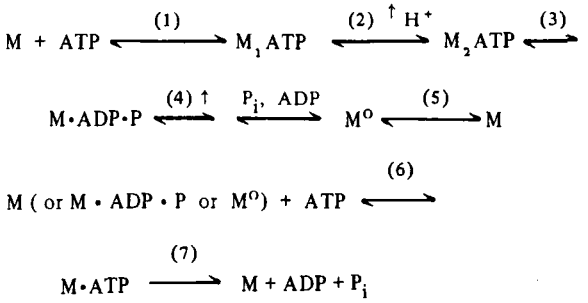
An independent activation mechanism is not unreasonable. If the same interaction between heads is present in the formation of a complex of HMM·Pr_2 as for HMM itself, the effective association constant of the second head with an adjacent G-actin residue could be quite small. The association constant of the SF-1 product intermediate (K_4) is only 10^4 to 10^5 M^{-1} at very low ionic strength, and it is considerably smaller in 100 m*M* KCl. The association constant for the second head of HMM·Pr_2 would be roughly 1 to 10 in the absence of interaction and less than 0.1 if the interaction is similar to the HMM case. Thus, attachment of the heads to different actin filaments could be more favorable for actin concentrations exceeding 10 to 20 μM. An investigation of the physical state of the system is necessary in order to interpret the kinetics of acto HMM ATPase at high actin concentrations.

There is also a glaring discrepancy between the K_M of acto-HMM and actomyosin ATPases. The apparent K_M for acto-HMM at low ionic strength is 5 to 10 μM, while values for actomyosin are 1 μM,[23] 0.2 μM,[38] and 0.24 μM.[180] The question is whether the large difference in K_M can be explained merely by the state of aggregation of the myosin at low ionic strength.

Mechanism of Myosin and Actomyosin ATPase of Tonomura

Tonomura and colleagues have made important contributions to the mechanism of myosin and actomyosin ATPase. It is appropriate to discuss these studies as a unit since all aspects of the mechanism have been investigated independently of work in other laboratories and the mechanism proposed by Tonomura is significantly different in some aspects. It is not possible to discuss this large body of evidence in detail, but the general results have recently been reviewed.[217,218] The importance of the early phosphate burst in the myosin mechanism was first recognized by Tonomura who showed the burst to be essentially stoichiometric.[102,214]

A kinetic scheme for myosin ATPase was proposed in 1969 based on the results of a series of ten papers.[216] Like any kinetic scheme, it has evolved with time and in its current form,[218] the mechanism is



The mechanism postulates two pathways for ATP hydrolysis — one involves a product intermediate state (Equation 3) and the other a substrate complex for which hydrolysis is the rate-limiting step (Equation 4, which is termed simple hydrolysis). Step 4, as in the Bagshaw-Trentham mechanism, includes stepwise dissociation of phosphate and ADP and the release of a proton at pH 8. The original scheme included an initial proton absorption step, a phosphorylated intermediate, and the reverse order of product release. These results were not confirmed by other laboratories and are omitted for simplicity in recent reviews.

A weakness of the original mechanism as formulated in 1969 was that the various intermediates and the order of the steps were inferred mainly from measurements of the apparent rate constants and through indirect arguments. The consequences of the scheme were not testable in the absence of information on the magnitude of the first-order rate constants. The quantities K_1 and k_2 at low temperature were obtained by stop-flow methods using H^+ release and ultraviolet difference spectra.[94] The constants are in agreement with results from other laboratories although the proton release was one per myosin rather than 0.6 ± 0.2, a range which would encompass all the results reviewed here.

In its original formulation, the mechanism appeared to be very different from what has been discussed so far because the two pathways were connected by a common intermediate M_1ATP, i.e., this intermediate was a branch point so that M_1ATP could go to M_2ATP with the release of a proton or could break down with the release of products. The scheme was formulated in terms of a single ATP site per myosin molecule since the phosphate burst was $1/4 \times 10^5$ g. In view of the uncertainties in myosin molecular weight, the failure to recognize the presene of impurities, and the disagreements in the number of binding sites for pyrophosphate and ADP which were current at the time, the choice of a single ATP site was not unreasonable. It was reported that the rate of release of a proton after hydrolysis or of the reaction products was much slower than the steady-state rate and, therefore, a second pathway was required.

The dependence of the steady-state rate on ATP concentration also did not fit a single Michaelis constant, and it was suggested that Equation 3 was dominant at very low ATP concentrations and Equation 4 at high ATP concentrations (>1 μM ATP). However, a branching mechanism with a common myosin substrate intermediate cannot explain this result. In addition, the scheme was inconsistent because proton release was both fast and stoichiometric for step 2 while k_7 is approximately the slow steady-state rate. As a consequence, the flux must be almost entirely through the product intermediate. A branching pathway was not retained in later formulations[217] and it is clear that the first part of the mechanism (Equation 3) minus step 5 is the Bagshaw-Trentham scheme. The conclusions were reached independently, and to emphasize the similarity and the contributions of Tonomura, it would be appropriate to refer to the scheme as the Bagshaw-Trentham-Tonomura mechanism. Much of the confusion and disagreement arises in adapting the scheme to the fact that myosin has two ATPase sites. In the 1974 review,[217] the possibility was still considered that only one of the two

SF-1 heads had ATPase activity, but Arata et al.[4] state that the two pathways may refer to different heads.

The state M° was introduced to explain the discrepancy between the rate of product or proton release and the steady-state rate, and also the small decrease in the amount of ADP bound to myosin after the initial transient.[95] It is suggested that product release generates M° which cannot bind ATP and recycle via equation 3; consequently, the amount of ADP bound in the M·ADP·P state will decrease during the first few turnovers before reaching the steady state. The discrepancy in the rate of product or proton release was not found by other workers[11,29,196,210] nor even by Inoue and Tonomura.[94] Furthermore, the introduction of M° is not consistent with the experimental results and the further contention that the difference spectrum or fluorescence enhancement is given only by the M_2ATP and M·ADP·P states.[94] After an initial transient, the signal should decay to a value determined by the ratio $k_5/(k_4 + k_5)$. This effect occurs as already discussed[11] but only at low temperatures where the rate of ADP dissociation is comparable to the steady-state rate. In a recent paper, it was concluded that M° binds ATP and gives a difference spectrum.[188] Consequently, there is no direct evidence for this extra state.

The strongest evidence for a second pathway is the finding that myosin contains as much as 1 mol of bound ATP in the steady state and the phosphate burst is one per myosin.[92] These results were indirectly confirmed by studies in other laboratories for the conditions of low temperature and ionic strength.[30,209] However, these results are explained by a single pathway (Equation 3) and a value of K_3 close to unity. The latter explanation is supported by the fact that the ATP does not exchange with ATP in the medium and is partly converted to products by a temperature-pH jump,[209] although the results are not quantitative. Moreover, there is a clear discrepancy at 20°C where other laboratories report a burst of 0.85 to 0.9 per head.[207,209] The reason for the discrepancy in size of the early burst and the amount of bound ATP at 20°C is not understood, but if correct this evidence still would not require a second pathway since it could be explained by a low value of K_3. A further consequence of the Tonomura scheme is the dominance of Equation 4 at moderate ATP concentrations. In it, hydrolysis is the rate-limiting step and little or no intermediate exchange is expected in ^{18}O experiments, yet an exchange of at least two and in some cases all three phosphate oxygens occurs and the rate of exchange is the correct magnitude to be determined by the reversal of step 3 in Equation 3.

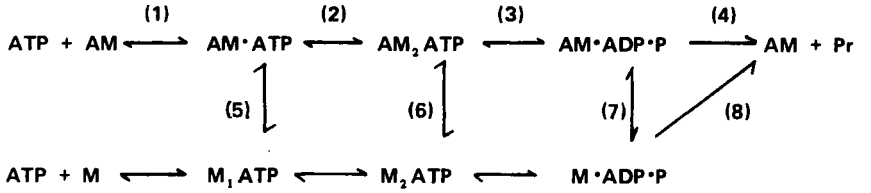
In summary, the extensive kinetic studies of Tonomura and colleagues independently established the main features of the myosin mechanism. The evidence for two different pathways occurring on the two heads of a single myosin is not conclusive and leads to disagreements with a variety of evidence from other laboratories. The scheme cannot be criticized simply because of disagreements in experimental results, but the requirement for internal consistency does restrict the deductions from the kinetic evidence.

The question could be settled by isolation of two SF-1 fractions which have the properties required by the mechanisms (Equations 3 and 4). Recently, Inoue and Tonomura[96,97] and Tawada[206] have reported the separation of SF-1 by interaction with actin at low ATP concentrations using ultracentrifugation or actin affinity columns. The two fractions have phosphate bursts of 0.8 to 0.9 and 0.2 to 0.3, respectively. Further characterization of the fractions is awaited with interest. SF-1 has also been separated into two fractions by differential actin binding by Winstanley et al.;[242] however, the fractions, SF-1A1 and SF-1A2, give the same phosphate burst.[90]

A different view is expressed by Morita and collaborators. Based on difference spectrum measurements, they concluded that only one half of the SF-1 molecules can give a spectral change when ATP or ADP is bound.[149,247] The phosphate burst is 0.46±0.06 per head for SF-1 and 0.65 per head for HMM.[248] In contradiction to Tonomura, the decay of the difference spectrum agrees with the steady-state rate and shows the same

pH dependence.[150] It is concluded that only one head per myosin contributes to the steady-state rate. Thus, the scheme favored by Morita is the Bagshaw-Trentham mechanism except that only one head of myosin and one half of the SF-1 molecules participate. The second head can bind nucleotides but apparently does not contribute to hydrolysis.

Tonomura has proposed two pathways for actomyosin ATPase, but the M·ADP·P intermediate participates in both pathways and the second part of the preceding mechanism (Equation 4) need not be considered in the discussion.[218]



This scheme is seen to be the one already discussed with the addition of step 8 and with different values for some of the rate constants. As already noted, there is no direct kinetic evidence for step 2. The scheme as presented is an irreversible model. At low actin concentrations, the system is essentially dissociated but the pathway of dissociation is primarily step 7, although step 6 may contribute at very high ATP concentrations. Reassociation must follow a path which is different from step 7 and occurs approximately at the steady-state rate of myosin ATPase. Presumably, step 8 is the dissociation of M·ADP·P to M and the reassociation of M with actin. The evidence for the mode of dissociation is the equal rates for the dissociation and the formation of a myosin intermediate state at low ATP concentrations.[92] Thus, the result is in agreement with Lymn and Taylor[122] and measures an apparent rate constant which cannot give evidence on the sequence of the steps. In view of the recent studies in which the first-order rate constant for the formation of M·ADP·P was clearly shown to be much smaller than the rate of dissociation,[31,193] their conclusion is incorrect.

At high actin concentrations, the dominant pathway is steps 1 through 4. The main evidence is an increase in the rate of acto-HMM ATPase over a range of actin concentrations in which the rate of reassociation of M·ADP·P with actin is independent of actin concentration and smaller than the rate of the ATPase[93] (a discrepancy of a factor of 2 can be obtained by reporting the steady-state rate per HMM rather than per site). The result contradicts the evidence of Chock et al.,[31] who found that the ATPase and recombination rates reach the same maximum value.

In the Tonomura mechanism, direct hydrolysis is the dominant pathway at all actin concentrations. The question to be settled is not whether direct hydrolysis can occur, but rather the extent to which it does occur. A direct pathway raises the obvious problem when applied to muscle contraction that the cross bridge does not dissociate, yet contraction is probably identified with step 4. Tonomura has proposed a complicated flip-flop model in which a myosin head alternately uses the mechanism of equation 3 for movement and the mechanism of equation 4 for cooperative dissociation (see Reference 7 for details). The two pathways and the complex acto-HMM scheme of Tonomura refer to an essentially irreversible dissociation model. The contribution of a second pathway previously discussed for acto-SF-1 ATPase arises when dissociation is considered to be reversible.

The Function of Sulfhydryl Groups

It is well known that two sulfhydryl groups per head will undergo preferential reaction with a variety of sulfhydryl reagents.[186] The groups, termed SH-1 and SH-2, have been characterized by the effect of modification on the Ca or Mg ATPase and the so-

called K-EDTA ATPase activity (the activity in the absence of divalent cations, but with K^+ as the presumed counter ion of the substrate). Reaction with iodoacetamide (IAA) or *N*-ethylmaleimide (NEM) activates the Ca or Mg ATPase by five- to tenfold and inhibits the K-EDTA ATPase. The degree of specificity depends on the reaction conditions but at low ionic strength and temperature, the reaction of one SH group per head gives maximum activation or inhibition.[178] The conclusion that the reaction is specific for a single SH group is often based on linear extrapolation of the curve of activity vs. moles of bound ligand to zero activity, which is not quite the same as reacting one SH group and obtaining complete inhibition. However, the reaction of 1.1 to 1.2 mol of SH groups per head can give essentially the complete effect. The fluorescent analogue of iodacetamide (IAEDANS) appears to be more reactive than the parent compound and complete inhibition was obtained with 1.1 groups reacted, and peptide analysis verified that 90% of the label was present in the SH-1 peptide.[40]

A second sulfhydryl group reacts preferentially in the presence of ADP,[178] and a fairly specific reaction can be obtained by blocking SH-1 with fluorodinitrobenzene, reacting with NEM, and removing the dinitro-phenyl group with dithiothreitol. Reaction of this sulfhydryl, termed SH-2, inhibits K-EDTA ATPase and slightly affects Ca ATPase.[175,183] The two sulfhydryl groups have been located in a single heavy chain peptide and are separated by ten residues.[50]

Earlier studies of modification of myosin SH groups were motivated by the idea that activation of myosin ATPase by the reaction of an SH group and by actin might have something in common. The rate-limiting step of myosin ATPase is a transition of the M·Pr** state, which determines the rate of release of products, and the modification of the SH-1 group increases the rate constant k_4, which accounts for the increase in steady-state ATPase.[196] The rate of product release is much faster in the AMPr state as compared to M·Pr**, and in this sense, the earlier ideas were apparently correct.

Recent studies by Burke, Harrington, and Reisler[22,174,175] have led to an intriguing proposal for the function of SH-1 and SH-2 groups and the mechanism of actin activation. It was suggested that the divalent metal ion (Mg or Ca) which is bound to the substrate or reaction products is held in a complex by the two sulfhydryl groups. Formation of the complex is prevented by reacting SH-1, which allows a more rapid rate of the release of products. Binding of actin presumably near the SH-1 site opens the complex and allows for the release of products. This proposal is consistent with a number of features of the mechanism. The rate-limiting step of myosin ATPase is dependent on a divalent ion and k_4 increases in the order Mg<Mn<Ca<K. ATP protects both sulfhydryl groups against reaction by NEM.[63] Actin is close to or alters the environment of SH-1 since actin also protects[40] and perturbs a spin label on it.[185,203] Recent studies have shown that the bifunctional derivative *N,N'*p-phenylenedimaleimide reacts with both SH-1 and SH-2 with complete inhition of Ca or K ATPase.[22] The two groups are within 12 to 14 A of each other, which is consistent with the peptide mapping of Elzinger. Furthermore, bridging of the SH groups produces a different conformation of myosin than reacting SH-1 alone or SH-1 and SH-2 separately. SF-1 or HMM with a modified SH-1 group binds to actin with an affinity at least as high as the normal enzymes.[69,133] The bridged disulfide derivative does not bind to actin, and the circular dichroism spectrum is altered in a similar fashion to that first described by Murphy[153] for the interaction with ATP. The authors suggest that the conformation of the bridged disulfide is similar to the postulated chelate formed in the M·Pr** state, hence, its low affinity for actin.

Certain criticisms have been made of this mechanism. The degree of activation of Mg ATPase by modification of SH-1 is much less than that produced by actin, but the authors suggest that at a physiological ionic strength the difference is small and the ATPase activities of both modified and actin activated myosin have the same de-

pendence on Mg concentration.[23] A weakness in the argument is the lack of precedence for thiol-metal complexes. In fact, the complex would have to be a weak chelator of Mg since the only strong Mg (Ca) binding site of myosin is located on the P-light chain which is dispensible for ATPase activity.[7,238] Attempts to measure the distance between the SH-1 group and the active site by placing a spin label on SH-1 and Mn at the active site using Mn ATP as the substrate gave negative results.[9] The distance is greater than 20 Å as interpreted by the usual coupling theory.

The reaction of SH-1 does more than simply increase the rate of product release. The rate of the hydrolysis step (k_3) at pH 8 is reduced tenfold and is independent of pH between 6.0 and 8.5.[196] The amplitude of the tryptophan fluorescence signal is reduced by at least one half,[184] and the rate process is now markedly biphasic[196] which allows the two-step fluorescence change to be directly observed. Thus, modification affects both the catalytic step and the change in environment detected by tryptophan fluorescence.

The modified enzyme is poorly activated by actin[151,152] both on an absolute scale and relative to the activity of modified heavy meromyosin; the degree of association at V_M is almost unmeasurable except at zero ionic strength. Also, the velocity of the shortening of actomyosin threads is reduced in proportion to the fraction of those heads reacting with NEM.[63] It is not clear that the kinetic evidence can be explained by a simple model. The statement that the modified enzyme is more "refractory" (Mulhern and Eisenberg) is not altogether helpful.

A different effect of modification of sulfhydryl groups has been obtained by Yount and collaborators using a purine disulfide analogue of ATP, 6,6′-dithiobis (inosinylimidodiphosphate). The molecule might be expected to bind to the active site since it is similar to AMPPNP. However, more than 2 mol are bound per head without blocking the binding of AMPPNP[226,227] for the analogue instead undergoes disulfide exchange with protein SH groups. Conditions were obtained which gave complete inhibition of K-EDTA ATPase and partial inhibition of Ca ATPase for the reaction of one SH group. In this case, the SH group is located in the alkali light chains A1 and A2 of striated muscle myosin or the corresponding A-light chain (LC-1) of cardiac myosin.[59,60] The cardiac light chain has three sulfhydryl groups, but the reaction was confined to the one group whose sequence environment is similar to the single SH group of the striated muscle light chain. The reaction of the A-light chain SH group blocks or at least reduces actin binding.[228]

The results of the two studies of SH groups need not be mutually exclusive. The formation of a bridged sulfhydryl ring on the heavy chain and the reaction of the A-light chain SH group may both affect actin binding, but possibly at different sites of contact.

Regulation of Actomyosin ATPase

Type 1 — Troponin Regulation

The most detailed evidence has been obtained for type 1 regulation, and a simple structural mechanism, the tropomyosin-shift model, qualitatively accounts for most of the observations. Demonstration of pairwise association of the components by binding studies[78,171] and association in paracrystals[125] is consistent with the geometry of the model shown in Figure 2. Ca binding to troponin C (TN-C) reduces the binding of TN-I to actin tropomyosin[79] and influences the interaction of TN-C with TN-T.[41a] A plausible model is provided by supposing that Ca binding to TN-C favors dissociation of TN-I from its actin-tropomyosin site, permitting tropomyosin to take the position in the actin groove which it normally occupies in the absence of the regulatory system. Presumably there are two possible positions for tropomyosin itself which correspond to the active and relaxed configurations, with the active position having the lower free

energy. From sequence considerations, McLachlen and Stewart[137] have suggested that there are two sets of seven sites along the length of tropomyosin which interact with actin residues and that a quarter turn rotation of tropomyosin would detach one set of seven sites and attach the other.

The complex of TN-I with actin-TM inhibits actomyosin ATPase in a one to seven actin mole ratio in the presence or absence of Ca.[167] This fact could be explained by TN-I stabilizing the relaxed conformation.[229] The model with opposite geometry in which the regulatory components are placed on the groove side of tropomyosin would also be consistent with the evidence.[123] In this case, the relaxed position is maintained by steric hindrance from the attachment of TN-I to actin.

The biochemical expression of the regulatory mechanism has been extensively studied (as reviewed by Weber and Murray),[233] and there is agreement on the general result. At a moderate actin concentration, the activity of the complete system actin-TM-TN can be reduced essentially to the activity of myosin by the removal of Ca. The activity ratio plus or minus Ca will depend on the conditions of assay, but a factor of 20 is obtained routinely. The complete system (A-TM-TN) will be referred to as regulated actin and the states with high or low ATPase activity as active and relaxed states.

The question is which step or steps in the actomyosin ATPase cycle are altered in the relaxed state. A rather obvious answer provided by the TM-shift model is that some or all myosin intermediate states are unable to bind to actin because the site is blocked by tropomyosin. This statement is translatable into chemical terms within the context of the reversible steady-state scheme (model III) by allowing the association constants K_2 and K_4 to be reduced in magnitude. An irreversible model with or without a refractory state does not provide for regulation in a simple way because the rate constant for the attachment of two proteins cannot be lowered arbitrarily. If no complex can form, the rate constants of association and dissociation refer to a collision intermediate and are determined by diffusion which again states that the attachment step is at equilibrium.

It is not clear whether a complex is unable to form because of steric hindrance or if a weak complex is possible using a part of the actin site. If we consider the simple case in which actin inhibition is not significant, the activity of the ATPase is characterized by K_M or K_4 and it is clear that reducing K_4 by a factor of 20 to 30 will account for the inhibition observed in solution studies. Inhibition could also be obtained if a complex forms, but the interaction between actin and myosin moieties is altered so that the rate of product release is not increased as compared to myosin, i.e., k_5 is reduced. The degree of association is unchanged in the simple scheme, but if there is actin inhibition it would be increased.

Surprisingly little has been done to measure V_M, K_M, and the degree of association of the inhibited system. Some earlier studies[47,162] indicated an increase in K_M, no change in V_M, and a lower association. The observed change in K_M was rather small in order to account for the degree of inhibition usually obtained. The system has not been properly investigated, and there are serious difficulties in the interpretation of the available experiments. A problem arises from the observations of Bremel, Weber, and collaborators. They found that at low ATP concentrations in the absence of Ca, the actomyosin ATPase activity remains high,[20] most likely because a fraction of the myosin which is not complexed with nucleotide is able to bind strongly to actin and displace tropomyosin from the blocking site. These results are explained by the presence of only two states — the relaxed state which does not bind myosin intermediates and the active state in which the TM has returned to the nonblocking position and the interaction with myosin intermediates is the same as in the active state. The evidence also indicates that this transition is cooperative. The explanation in terms of only two states may be too simple, but the interpretation of the results emphasizes that the binding of myosin or Ca both tend to turn on ATPase activity.

Transient kinetic studies have contributed little except to verify the switching off of regulation by SF-1. The rate constant for the association of SF-1 with regulated actin minus Ca is slightly smaller than with Ca or with pure actin present,[240] but it is not clear what this experiment measured. If SF-1 does not bind to the relaxed state, a lag might be observed for switching to the active state. Evidence on this point is unsatisfactory. Dissociation of the complex of regulated actin and SF-1 by ATP was also not Ca dependent, but the initial state was presumably the active state. The recombination of M·Pr with actin in a single turnover experiment failed to show regulation. However, this could again be explained if the initial state of acto-SF-1 is active, the recombination begins before all SF-1 is removed, and the actin-TM-TN complex is still in the active state. Regulation was demonstrated in preliminary experiments in which the complex of HMM product maintained by a phosphocreatine-creatine kinase system was mixed with regulated actin. A distinct lag in complex formation was observed in the absence of Ca.[107] The experiment has been repeated using double mixing to generate the SF-1·Pr complex with similar results. The SF-1·Pr state combines slowly with actin after a lag.[240] Qualitatively, the experiment demonstrates a weak association of M·Pr with the relaxed state and the slow recombination may arise from the displacement of tropomyosin by the free SF-1 generated through the decay of the product state. Quantitatively, the results are not interpretable except to show that the apparent rate constant of association of M·Pr, $k'_4 = K_4k_5$, is very much reduced. A low value of K_4 is sufficient to explain the results.

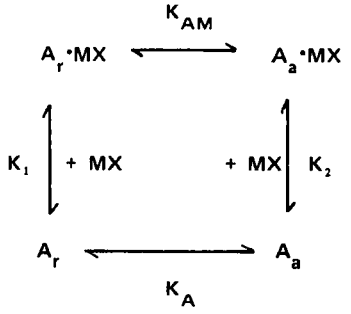
Further studies[19] have shown that the system may not be described by two actin-TM-TN states. The ATPase activity of regulated actin SF-1 plus Ca is much larger than pure actin SF-1 at low ATP concentrations, and the rate passes through a maximum with increasing ATP and falls to approximately the value of pure actin at high ATP. (This effect is referred to as substrate inhibition.) Furthermore, the rate per actin site and the optimal ATP concentration depends on the SF-1 to actin ratio. At a very low ratio of SF-1 to actin, the ATPase activity of the regulated system plus Ca is lower than pure actin at all ATP concentrations. The rate increases with an increase in the SF-1 to actin ratio, and a double reciprocal plot of rate vs. SF-1 obtained from data at high ATP concentrations shows positive curvature but with the same V_M as for pure actin. This evidence requires a decrease in K_M (an increase in K_4) with an increase in the concentration of SF-1·Pr and, thus, in the fraction of actin sites associated with myosin intermediates.

The same effect occurs with actin tropomyosin except that the decrease in rate begins at a lower ATP concentration.[19,187] At high ATP concentrations, tropomyosin inhibits actin activated ATPase by 60%[41] as first demonstrated by Katz[103] and TN-I increases the inhibition to 85%. Even with pure actin the SF-1 to actin ratio with SF-1 in excess influences the kinetic behavior. A double reciprocal plot of the rate per actin residue vs. SF-1 (the extrapolation to infinite SF-1 discussed previously) gives a K_M which is invariant but a V_M which decreases with increasing actin concentrations.[48] Bremel et al.[19] have suggested that the significant parameter is the degree of association of SF-1 or SF-1 intermediate states with actin, since this quantity increases at low ATP or high SF-1 to actin ratios. The results imply that the binding of SF-1 to actin activates the ATPase presumably by altering the structure not only of the actin-TM-TN complex but of the actin-TM as well.

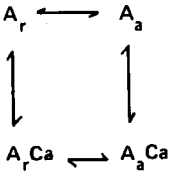
There is some evidence from physical measurements that the binding of SF-1 to pure actin produces a cooperative change in structure. The flexibility of actin is reduced by SF-1 as measured by saturation transfer EPR spectroscopy.[213] Only 0.1 of SF-1 per G-residue are required for the maximum effect. TM decreases actin flexibility,[160] although the interpretation of laser light scattering measurements has been challenged by Carlson and Fraser[25] and no change in flexibility was observed by Thomas et al. by the EPR method. These studies indicate that binding myosin and possibly tropom-

yosin affect actin structure, and in the case of myosin, the effect is cooperative. The relation of these changes to structural changes observed by X-ray diffraction and optical reconstruction is not known.

A kinetic model to explain "potentiation," the higher activity of the complete system at low ATP concentrations, was given by Bremel et al.[19] Squire[201] has also outlined a "rate" type of model to explain type 1 and 2 regulation which is similar in its basic postulates to the proposal of Bremel et al. However, as already discussed, a rapid equilibrium mechanism probably is a better description of the system and has the distinct advantage of making the thermodynamic relations explicit. Consider the case in which there are only two actin-TM states (the generalization to three states is straightforward). Let A_a and A_r refer to active and relaxed conformations, respectively, of actin-TM or actin-TM-TN complexes and let MX stand for a myosin state (M or M·Pr). In the absence of Ca, the association-dissociation equilibria of regulated actin can be represented by the following:



where K_1 and K_2 are the association constants of MX with the relaxed and active states. A_r is the lower energy state, $K_A<1$, and $K_A = [A_a]/[A_r]$. Regulation is described by a lower value of the association constant K_1 as compared to K_2. Since $K_1K_{AM} = K_2K_a$, it follows that $K_{AM}>K_A$. Hence, the binding of MX shifts the system to activated states and MX turns on the ATPase in the absence of Ca. If K_1 is very small because the binding of MX is blocked by steric hindrance in the A_r state, the thermodynamic argument is still valid. In the presence of Ca, there are eight states but the treatment is similar. If the equilibrium is only partially to the side of A_a states in the absence of MX, MX will shift the system to a larger fraction of A_a states and "potentiate" the ATPase activity. Furthermore, from the box



it can be seen that since the activation by Ca must shift the equilibria to A_a states, it follows that Ca is more tightly bound to A_a. It is not necessary to assume that Ca is more tightly bound to TN when TM is out of the groove[201] because this is a thermodynamic consequence of the structural model. Also a shift to A_a states produced by binding myosin will increase the apparent affinity of the system for Ca. Thus, the various effects, potentiation, and the turning off of regulation by myosin and myosin intermediates and the increase in Ca affinity are qualitatively explained by a simple model in which there are at least two actin-TM states.

The results are still difficult to reconcile with the simple TM-shift model, although it may be premature to decide whether the fault rests with the kinetic scheme or the TM-shift model. If the actin is simply a support for tropomyosin (which can occupy

either of two positions on the actin), one expects the ATPase activity of A-TM-TN plus Ca or A-TM to be the same as pure actin. However, it is slightly lower for regulated actin and 60% lower for A-TM when measured at high ATP concentrations. It could then be assumed that, in solution, the two positions of TM in the actin-TM complex are almost equally probable, whereas it is essentially in the active position in the A-TM-TN·Ca state. An increase in ATPase activity is readily explained by the displacement of tropomyosin by the binding of SF-1 or its intermediate complexes. Yet at low ATP concentrations, the ATPase activity relative to pure acto SF-1 is higher for A-TM and A-TM-TN·Ca; this is not explained if tropomyosin has simply shifted to a position in which it does not interact with myosin intermediates. Gillis, O'Brien, and collaborators[158] have shown that actin-TM can exist in two states distinguished by a change in pitch of the actin helix and that a change in pitch also occurs on binding Ca to regulated actin. It is necessary to consider the possibility that the conformation of actin residues as well as the position of tropomyosin may be different in the active and relaxed states.

Type 2 Regulation

Szent-Gyorgyi, Kendrick-Jones, and collaborators have examined the regulation of actomyosin ATPase in a large number of species including representatives of the major phyla.[112] Type 2 (myosin-linked) regulation is found in a variety of invertebrates and has been most thoroughly studied in molluscs. Myofibrils contain no troponin and pure myosin plus actin or actin tropomyosin is a Ca-regulated ATPase. The myosin contains 2 mol of P-light chain and 2 mol of a light chain analogous to the A-light chain (termed SH-light chains since they contain cysteine while the P-LC does not, in the case of scallop).[104] One mole of P-LC is removed by treatment with EDTA without any loss in ATPase activity (hence its name EDTA light chain), but the second is only partly removed by extensive EDTA treatment. The second P-LC is released by DTNB (the DTNB light chain), but its removal leads to an irreversible loss of ATPase activity. The two P-light chains appear to be identical and analogous to the P-LC of vertebrates. Having considered myosin to be a dimer, the difficulty in removing the second light chain requires an explanation (the second P-LC is also more difficult to remove from rabbit myosin).

The myosin has two Ca binding sites located on the P-light chains as is the case with rabbit myosin, but the affinity is roughly ten times larger for the scallop binding site. The removal of a single light chain with EDTA "desensitizes" the myosin such that the actomyosin ATPase is not inhibited in the absence of Ca. The light chain can be rebound in the presence of Mg with the restoration of Ca sensitivity.[205]

The SF-1 obtained from scallop myosin is not Ca regulated even though the P-LC appears to be intact. The light chain of SF-1 has been removed by EDTA and replaced with a light chain obtained from myosin; thus, the failure to obtain regulation cannot be attributed to a damaged light chain.

Ca sensitivity can be restored to scallop myosin lacking one P-LC by binding the P-LC of chicken gizzard myosin. The rabbit P-LC restores Ca sensitivity to densensitized actomyosin and myofibrils but not to pure myosin. Furthermore, the gizzard light chain combined with scallop myosin will bind Ca while the rabbit light chain does not, even though it does restore Ca regulation to the actomyosin.[104] Note that Ca regulation does not require tropomyosin, although the activity of the ATPase is higher with TM present.

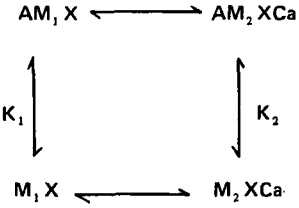
These studies raise interesting questions on the mechanism of regulation and on cooperativity between myosin heads. To date, there are no detailed kinetic studies of myosin-linked actomyosin ATPase. The obvious mechanism is a blocking of the actin binding site on the myosin by steric hindrance from the light chain or a change in

conformation at the actin binding site induced by interaction with the light chain. The binding of Ca to the light chain relieves the inhibition.[104]

It has also been suggested that there is cooperativity between heads because the presence of two regulatory light chains is necessary to maintain the relaxed state. The failure of regulation when only one light chain is present in myosin is not unexpected since the unregulated head would interact with actin. The ATPase activity of the actomyosin appears to be only slightly less than that when Ca is present. Measurements of K_M and V_M are not available and it is not clear whether the regulated head has been "turned on." Considering the discussion of acto-HMM ATPase, this question might be difficult to settle. The restoration of regulation by rabbit P-LC which does not bind Ca is also puzzling, but since it occurs only with actomyosin or myofibrils, it has been suggested that the conformation of the P-LC has to be altered by interaction with actin. Thus, the rabbit light chain may be induced to take up the conformation corresponding to the blocking state and, being unable to bind Ca strongly, it remains in the blocking state. The other light chain, unblocked by Ca binding, is sufficient to activate the actomyosin ATPase. The failure to obtain regulation with SF-1 is the strongest evidence for cooperativity, and further studies on the molluscan system may be of importance in understanding regulation in general.

Muscles showing myosin-linked regulation still give changes in X-ray diffraction patterns similar to those of vertebrate muscles attributed to the TM shift in the latter case. The intensity changes are smaller, and it has been proposed[163] that tropomyosin in the relaxed molluscan muscle is farther out of the groove than it is in the active state but not as far out as in the vertebrate (troponin) type of structure. Activation would then include a displacement of TM by the binding of myosin intermediates.[201] The proposal is clearly similar to the explanation of Bremel and Weber of the activation of vertebrate actomyosin ATPase by myosin binding. It is also a post hoc explanation which saves the phenomena.

Squire[201] has emphasized that the X-ray evidence does not require the position of TM to be fixed since a large temperature factor has little effect on model calculations. The tropomyosin may be envisioned as jumping between the two stable positions, in and out of the groove. The ability of myosin to turn on or potentiate ATPase activity in either type of regulation could be explained by transient availability of the binding site. The same considerations apply here as in the vertebrate case. However, regulation in solution is obtained with pure actin and could, therefore, be explained by a model in which there are two myosin states, M_1X and M_2X, and only one actin state. The M_2 state is favored by binding Ca.



Regulation requires $K_2 >> K_1$. This system would not show a cooperative activation of ATPase activity by binding myosin. The X-ray evidence suggests there are two actin-TM states; consequently, myosin-linked regulation should involve both a change in myosin conformation and thin filament structure. A crucial question is whether scallop actomyosin shows the same effects as rabbit actomyosin — those of a potentiation of the ATPase in the presence of Ca and activation in the absence of Ca by binding of myosin or myosin intermediates. According to some preliminary results (cited by Szent-Gyorgyi et al.),[205] activation by myosin does not occur. But, Ebashi et al.[42] refer to evidence for a sharp transition with increasing ATP concentration in the absence

of Ca. It might be of interest to try to demonstrate myosin activation by binding a nondissociable form of myosin.[166] The discussion indicates that a relatively simple model along the lines suggested by Weber or Squire accounts for many of the observations, although a clear explanation of the way in which the type 2 system is turned on will require further evidence.

Double regulation, the presence of both Ca-regulated myosin and regulated actin, occurs in many higher invertebrates,[112] but it has not been demonstrated for a vertebrate actomyosin. The functional test of Lehman and Szent-Gyorgyi gives positive evidence for double regulation with vertebrate myofibrils, according to Lehman.[111]

Margossian et al.[128] have observed that the association constant of rabbit SF-1 to actin is reduced tenfold by the binding of Ca to the P-LC. Also, the removal of one half of the P-light chains with DTNB decreases the Ca sensitivity of actomyosin ATPase.[238] Some Ca function of the P-LC is apparently retained in vertebrate myosin, but it is not clear that these observations have physiological significance.

Type 3 Regulation

The recent studies with smooth muscle indicate that myosin-linked regulation does occur in the vertebrates if not in the striated muscles. However, the details of the mechanism may be quite different from the molluscan type. A different conclusion has been reached by Ebashi, who considers that regulation is actin linked but requires a protein factor different from troponin.[42] The two proposals will be considered in turn.

Gizzard actomyosin or "myofibrils" do not contain detectable amounts of a protein that can be identified with troponin, but the freshly prepared actomyosin is a Ca-sensitive ATPase.[198,199] Gizzard actomyosin passes the actin competition test of Lehman and Szent-Gyorgyi[112] for the presence of myosin-linked regulation — the addition of rabbit actin does not activate the ATPase in the absence of Ca. The degree of activation of the actomyosin with increasing calcium concentration parallels phosphorylation of the preparation[3,199] and phosphorylation is confined to the 20-kD light chain (P-LC) of the myosin.[57] Phosphorylation of the purified myosin by a kinase in the presence of Ca activates the actomyosin reconstituted with rabbit actin. The dephosphorylated myosin-actin system is not activated in the presence or absence of Ca.[26] The rate of phosphorylation of the myosin is rapid and may precede the activation of the actomyosin ATPase.[197]

This set of observations favors strongly the argument that phosphorylation of the P-LC is necessary for the activation of the actomyosin ATPase. The important question is whether it is sufficient. Since the kinase requires Ca, actomyosin ATPase activity could be controlled by phosphorylation and dephosphorylation by the kinase and a phosphatase as was first proposed by Sobieszek and Small. An alternative is that phosphorylation is required for the binding of calcium to be effective in removing the inhibition by the P-LC. Proof that Ca is no longer required by phosphorylated myosin in a purified system is not yet available.

The parallels with the type 2 system are striking. Phosphorylation in one case and Ca binding in the other act by removing inhibition by the light chain. The papain SF-1 of gizzard lacks the P-LC and is not calcium sensitive, but the acto-SF-1 ATPase is active and, thus, analogous to scallop myosin in its behavior. Thus, a provisional interpretation is that the P-LC inhibits activity, and activation is obtained upon the removal of the light chain or by an alteration produced by the phosphorylation or Ca binding in the two systems. There is little kinetic evidence available except for the unregulated gizzard SF-1. The intermediate steps in the SF-1 and acto-SF-1 ATPase are similar to the rabbit scheme except that their values for k_3 and k_5 are smaller.[134,135]

A protein kinase and phosphatase specific for myosin appear to be present in various muscles, and the properties of the enzymes of rabbit striated muscle have been deter-

mined by Perry and collaborators. In the striated muscle system, purified enzymes have been used[168] and the results are quite clear: Phosphorylation of the myosin has no effect on the K_M and V_M of actomyosin ATPase.[145] In the one nonmuscle myosin that has been properly characterized, the platelet myosin, activation of the actomyosin ATPase requires phosphorylation of the myosin by a protein kinase.[1,2,37]

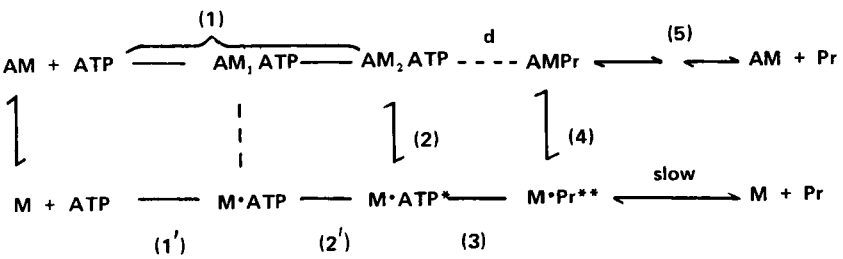
A different interpretation of regulation in smooth muscle has been proposed by Ebashi.[42,43,140] From the "natural tropomyosin" fraction also rich in protein kinase, Ebashi has obtained a pure protein factor of peptide chain weight 80 kD, which activates a reconstituted system of chicken gizzard myosin, actin, and tropomyosin. Here, rabbit skeletal actin and tropomyosin do not replace the corresponding chicken proteins in the reconstituted system, while in the studies of activation of phosphorylated myosin by other workers, rabbit actin was satisfactory. On this basis, the actin competition test for myosin-linked regulation would not be applicable. Phosphorylation of the gizzard myosin by light chain kinase did not activate the ATPase unless the 80-kD factor was also present, and activation was obtained without phosphorylation in the presence of the factor. However, full activation occurred at a ratio of actin to 80 kD factor of 100 or more. The results tend to suggest that the factor may be an enzyme which alters gizzard actin, but the preliminary nature of the results does not permit a conclusion to be drawn.

Function of the A-light Chain

Removal of the A-light chain leads to the complete loss of ATPase activity of myosin, and until recently, this was where the experiment ended. The separation of SF-1 into two fractions with different light chains (SF-1, A1 and SF-1, A2)[236] showed that the two have nearly identical kinetic properties.[207] However, when they are combined with actin, the K_M of the ATPase is five times larger and the V_M two times larger for SF-1, A2 as compared to SF-1, A1. The interchange of A-light chains between fast and slow skeletal myosins alters the actomyosin ATPase in the direction that would be expected if the light chain were a major factor in determining the rate of actomyosin ATPase.[224] Reaction of the A-light chain sulfhydryl group decreases the binding to actin.[228] Comparison of the rate constants for the intermediate steps in SF-1 and acto-SF-1 from fast and slow striated, cardiac, and smooth muscles showed that the major differences are in the steps which involve interaction with actin.[134,135] These observations suggest that the A-light chain interacts with the ATP site and the actin site and is a strong determinant of the rate of the actomyosin ATPase.

CONCLUSIONS AND CONJECTURES

An attempt to critically review the problem of muscle contraction must stress the difficulties and discrepancies, when, in fact, considerable progress has been made in our understanding of the mechanism. A kinetic scheme applicable to acto-SF-1 at least up to moderate actin concentrations, has been formulated and is supported by fairly detailed evidence. Some of the studies are very recent and further revisions may be necessary.



Asterisks are omitted from actomyosin states to avoid confusion since equal fluorescence enhancement need not be an index of conformation. The mechanism is termed "the scheme" because it is based on work from several laboratories. The rate constant k_3 is essentially the same measured by fluorescence for M or AM as required by the scheme. Previous difficulties may be overcome by reconsideration of the fluorescence evidence which indicates that the formation of M·ATP* is much faster than the observed fluorescence signal. Further information is clearly needed on step 2′.

There is no direct evidence for a conformation change prior to dissociation (AM_2ATP state), and the second actomyosin substrate state is included by analogy with the myosin pathway. The initial binding of ATP to AM appears to have a low association constant, one comparable to K_1', and it is probable that some change in structure must occur before dissociation of the actomyosin complex. If there were a single AM-ATP state, the dissociation constant K_2 would have to be extremely small ($<1\ M^{-1}$) by the usual thermodynamic argument. $AM_2 \cdot ATP$ may not be detectable if the rate limiting step in dissociation is a conformational change followed by rapid dissociation.

The steps in product release from AM are also unclear. It might be supposed that there is a sequential dissociation as in the myosin sequence. It is known only that the rate constant of ADP dissociation from actomyosin is much faster than the maximum rate of hydrolysis and also faster than the corresponding myosin step.[239]

A further myosin intermediate may be necessary to account for the evidence at high actin concentrations. As formulated by Eisenberg and collaborators M·Pr** is a "refractory state" which binds very weakly to actin and is slowly converted to a second myosin-product intermediate M·Pr‡. Hence, step 4 would have to be expanded into two steps. The refractory state was introduced originally to explain the low association of acto-SF-1 ATPase at high actin concentrations for which the steady state rate appears to have reached a maximum value. The degree of association is partly explained by the relative magnitudes of the rate and equilibrium constants of the scheme. In addition, V_M ($A \rightarrow \infty$) is a poorly defined quantity since the kinetic scheme predicts that the rate will pass through a maximum value whether or not a refractory state is present. Nevertheless, there is a discrepancy between the measured association and the value calculated from available kinetic constants. The discrepancy should be larger at 20°C, and a complete set of transient and steady state measurements might settle the question. The problem is important, since it was shown by Barany[13] that V_M correlates with the speed of shortening of muscle, yet we are still not clear as to whether V_M is determined by the rate of release of products from actomyosin (k_5) or by a transition of the myosin. Also, some contribution to the steady state ATPase by the direct hydrolysis pathway can not be ruled out at high actin concentrations.

A general property of ternary complexes such as AM·L, where L is ATP, ADP, AMPPNP, PP_i etc., is that they have a larger dissociation constant of the protein complex in comparison to that of AM. Thus, all myosin intermediate states are refractory, although some are more refractory than others. As a general rule, ligands and actin have antagonistic effects on the association at their respective binding sites. This may be explained by the conformation changes induced at the actin site by ligand and vice versa.

Transient studies on the kinetic mechanism of regulated actomyosin have shown little progress. Steady-state kinetic studies, particularly those by Weber and collaborators suggest that actin can also undergo conformational changes in the ATPase mechanism. The effects of TM-TN or TM alone on the ATPase, the so-called substrate inhibition of actomyosin, and even some effects in pure actin-myosin systems are most easily explained by the effect of myosin binding on actin structure.

In the rotating cross-bridge model (Figure 1), two acto-SF-1 states are postulated in which the head is bound in different orientations. Emphasis has been placed on the

myosin moiety and the effect of substrates or product on determining the orientation state. It is likely that such states occur for the acto-SF-1 complex itself, and it would be surprising if the change in conformation were restricted only to the myosin part.

A primary question is whether the kinetic scheme in solution can be used as the basis of a muscle contraction model. It could be supposed that the contraction mechanism is essentially a two-head reaction involving cooperative, flip-flop, or other mechanisms in which the heads have different functions. The behavior of acto-HMM ATPase presents several problems, and if the mechanism is complex, it is not deducible from the available evidence. A contraction model which is based on the acto-SF-1 mechanism would then be fundamentally wrong. In opposition to this view, it is known that superprecipitation occurs with a single-headed myosin.[126] Recently, Crooks and Cooke[34] have prepared contractile filaments from actin and myosin for which the tension and contraction velocities are comparable to muscle when corrected for the much lower protein concentration. Single-headed myosin exerts essentially the same tension on a per head basis.[33] The effect of inactivation of heads by a sulfhydryl reagent on the velocity of shortening of actomyosin threads is also consistent with a mechanism in which the heads are independent. While it might be argued that two heads may be necessary in order to increase the efficiency, the basic contractile event can occur with a single head. The kinetic evidence on acto-HMM ATPase is compatible with independent activation of the two heads and even with interaction with different actin filaments at high actin concentrations.

Thus, it is reasonable to consider one-headed contraction models based on the acto-SF-1 ATPase mechanism. The objective is to collect a set of rate and equilibrium constants for the steps in the mechanism at the approximate physiological conditions.[241] A provisional set of equilibrium constants is shown in Figure 5. (This follows the suggestion of J. Pringle that a cyclic mechanism be illustrated by a circular diagram.) The values are the order of magnitude estimates for the conditions of 100 m*M* KCl and 10 m*M* $MgCl_2$ at pH 7 and 20°. The product of equilibrium constants for any complete hydrolysis cycle is 10^5M, the value of the equilibrium constant for ATP hydrolysis at the chosen conditions.[176] The individual values for the myosin cycle are probably correct to an order of magnitude. The difference between the association constants of ATP and ADP is probably less than a factor of 10. Recent studies indicate that the ATP constant has been overestimated, consequently, it is assigned a lower value than the ADP constant. Current work on the ADP reaction may require a reduction in the ADP constant as well, but the published value is used in the diagram.[11]

Actomyosin intermediates corresponding to each myosin intermediate state are included in the diagram, although the states $AM_1 \cdot T$ and $AM \cdot ADP$ have not been characterized in kinetic experiments. The value of the association constant of M·Pr** with actin assumes a rapid equilibrium model and the value of the MT* association constant is a rough guess as these two association constants could be equal. The association of myosin-ADP states with actin is 10 to 100 times weaker than for myosin.[15,69,239] The values for M·ADP and M·ADP* have not been determined separately, and the ratio could be larger than ten although the values used here are consistent with the available evidence.

It is clear that further studies are required, particularly on the partial reactions involving ADP, but allowing for the uncertainties in the data, the diagram illustrates some general features of the reaction. The association constants of myosin intermediates with actin probably increase as the cycle is traversed from MT* to M. The largest free energy drop in the myosin cycle (9.5 kcal) is the step from MT to MT*, which is necessary to produce dissociation. The free energy change in the hydrolysis step is small for myosin and probably somewhat larger for actomyosin, although the latter step is not observable and the value depends on the ratio of the association constants K_2 and K_4.

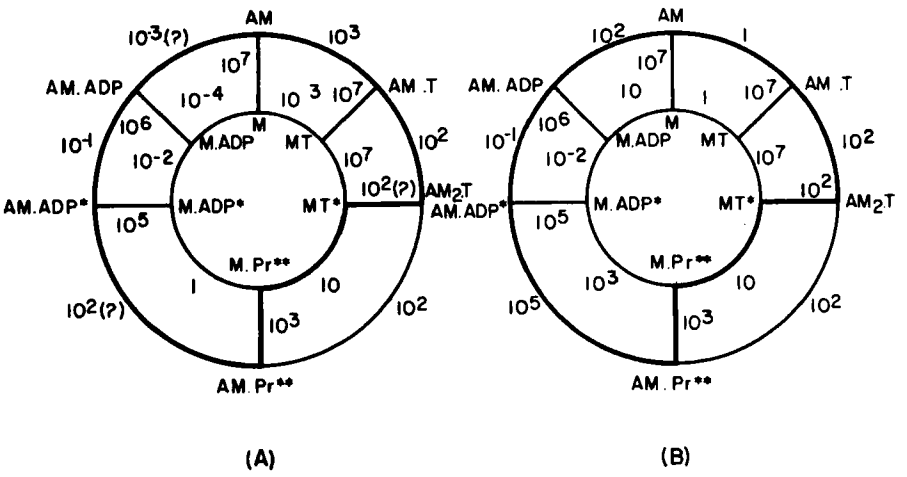
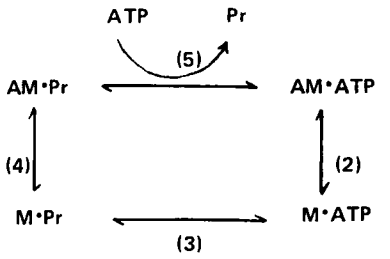


FIGURE 5. Provisional assignment of equilibrium constants for the acto-SF-1 cycle (100 m*M* KCl, pH 7, 20°). A. Values for radial steps are association constants (M^{-1}) for myosin intermediates with actin. The values for other second-order reactions are association or dissociation constants defined by a clockwise cycle. Values are order of magnitude estimates or guesses (denoted by a question mark). The states $AM_1 \cdot T$ and $AM \cdot ADP$ are included by analogy with the SF-1 cycle. Standard free energy differences between pairs of states are $-\Delta G° = RT\ln K$. B. Effective equilibrium constants K_* for the conditions [ATP] = 10^{-3}M, [ADP] = 10^{-5} *M*, and [P_i] = 10^{-3} M which roughly correspond to concentrations in muscle. All equilibrium constants around inner or outer circles are dimensionless; the basic free energy differences between states[3] are given by $-\Delta G^b = RT\ln K_*$. The heavy line in the diagram is the dominant cycle proposed by Lymn and Taylor[107] and Chock et al.[79].

The heavy line is the main pathway proposed by Lymn, Taylor, and Eisenberg and their collaborators; however, direct hydrolysis could make a small contribution. The energy relations for the steps in the cycle are illustrated in Figure 5B. The effective equilibrium constants are calculated for ATP, ADP, and P_i concentrations of 10^{-3} M, 10^{-5} M, and 10^{-3} M, respectively, as a rough approximation to the physiological concentrations in muscle. Thus, for steps involving substrate or products the ligand concentration is included in the equilibrium constant. For example, the reaction M + T ↔ M·T has an equilibrium constant K; $K_{eff} = K[T] = M \cdot T/M$. All reactions around each circle are now pseudofirst order and the basic free energy change for each transition is $-\Delta G^b = RT\ln K_{eff}$. If the heavy line is taken to be the major pathway, the performance of work is probably associated with the series of transitions from AM·Pr** to AM. A maximum of 60% of the free energy drop is available in this segment of the pathway. If work were derived from the direct hydrolysis of ATP, only 10 to 20% of the free energy would be available in the step from $AM_2 \cdot T$ to AM·Pr**.

A general treatment of coupled reactions in a lattice has been fully described by T. Hill[75-77] and some calculations have been made, although it is not yet clear that a model using a reasonable set of rate constants would be satisfactory. As discussed in the section on structural studies, a vital piece of evidence is still lacking. It is assumed that the cross bridge attaches preferentially at angle θ_1 and makes a transition to θ_2. The angles are often assumed to be 90 and 135°, but, to date, there is no direct evidence for this. Studies with analogues (AMPPNP and ADP) are suggestive[55,118,132] because they can be interpreted as evidence for an attached ternary complex in which the head makes an angle other than one of 135° with the thin filament. Kuhn has shown that AMP PNP can be carried around a Carnot cycle in which work is done by association and dissociation of the ligand. The energy is obtained from the ligand concentration ratio.[110] These results are consistent with two or more preferred angles of attachment for nonhydrolyzed ligands but are still not a satisfactory proof of rotation in the cycle.

The complete set of states for the dominant pathway in Figure 5 need not be used in model calculations. The minimum realistic model should include two attached states and two detached states. Since the ATP concentration is large, the rate of ATP binding to AM is much larger than product release and an approximate scheme is



The numbers refer to the steps in the acto-SF-1 scheme, and the cycle is running clockwise.

A model can be set up with the assumption that the AM · Pr complex has a minimum free energy at angle θ_1, and either AM or some other intermediate state has a minimum at θ_2. The consequences of this model have been discussed by Hill using plausible assumptions. The primary assumption deals with the lattice effect. In the lattice a complex can be formed with an actin site displaced by a distance x from the position of minimum free energy as measured along the filament axis. If K_4° is the association constant at the position of minimum free energy, then $K_4(x) = K_4^\circ \exp(-f_4/RT)$, where f_4 is the strain energy of the complex at x. In the simplest case, $f_4(x) = 1/2\alpha x^2$ provided that the deformation is treated as a Hooke's law spring, where α is a constant defining the compliance of the spring. More general cases are considered by Hill. In the same approximation, $K_2(x) = K_2^\circ \exp(-f_2/RT)$, where $f_2(x) = 1/2\ \beta(x-a)^2$. The distance between free energy minima, a, is determined by the geometry of the system, but it also can be considered roughly as the distance moved along x for the rotation of a cross bridge from θ_1 in the AM · Pr state to θ_2 in the AM state. The question of whether the elastic term should be treated as a "spring-like" deformation of a part of the cross bridge[91] or a broad angular free energy distribution of the complex is discussed in detail by Eisenberg and Hill.[46]

Calculations for a four-state mechanism with rate constants obtained from solution kinetics have not been published and the computational problem is formidable. The rate constants k_3, k_{-3}, and k_5° are obtained from kinetic studies. The quantities K_2° and K_4° are not equal to the association constants K_2 and K_4 as measured in solution, but their values should correspond to association in the absence of lattice constraints and one would expect K_2°/K_4° to equal K_2/K_4. The task of determining a set of constants is still incomplete and some assumption has to be made regarding the transitions between AM · Pr and AM · ATP. Computations will not be discussed here, but a proper test of the model requires that the physiological properties of muscles with different contraction velocities be predicted from the sets of rate constants obtained from the corresponding actomyosins.

There seems to be no reason to doubt that a mechanochemical mechanism will work "in principle" since Chen and Hill[28] have shown that the linear case of the general model in which the substrate and product concentrations are close to equilibrium satisfies the reciprocal relations of irreversible thermodynamics. It appears likely that a rotating cross-bridge mechanism can provide a satisfactory explanation of energy transduction in muscle, but, to date, there is no proof that the cross bridge rotates.

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REFERENCES

1. Adelstein, R. S., Chacko, S., Barylko, B., Scordeles, S. P., and Conti, M. A., Role of myosin phosphorylation in the regulation of platelet and smooth muscle contractile proteins, in *Contractile Systems in Non-Muscle Tissues,* Perry, S. V., Margrath, A., and Adelstein, R. S., Eds., North-Holland, Amsterdam, 1976, 153.
2. Adelstein, R. S. and Conti, M. A., Phosphorylation of platelet myosin increases actin-activated myosin ATPase activity, *Nature* (London), 256, 597, 1975.
3. Aksoy, M. O., Williams, D., Sharkey, E. M., and Hartshorne, D. J., A relationship between Ca sensitivity and phosphorylation of gizzard actomyosin, *Biochem. Biophys. Res. Commun.*, 69, 35, 1976.
4. Arata, T., Inoue, A., and Tonomura, Y., Standard free energy changes for formation of various intermediates in the reaction of H-meromyosin ATPase, *J. Biochem.*, (Tokyo), 77, 895, 1975.
5. Armitage, P. M., Tregrear, R. T., and Miller, A., Effect of activation by Ca on X-ray diffraction pattern of insect flight muscle, *J. Mol. Biol.*, 92, 39, 1975.
6. Bagshaw, C. R., The kinetic mechanism of the manganous ion-dependent adenosine triphosphatase of myosin subfragment I, *FEBS Lett.*, 58, 197, 1975.
7. Bagshaw, C. R., On the location of the divalent metal binding sites and the light chain subunits of vertebrate myosin, *Biochemistry,* 16, 59, 1977.
8. Bagshaw, C. R., Eccleston, J. F., Eckstein, F., Goody, R. S., Gutfreund, H., and Trentham, D. R., The magnesium ion dependent adenosine triphosphtase of myosin, *Biochem. J.,* 141, 351, 1974.
9. Bagshaw, C. R. and Reed, G. H., Equilibrium complexes of subfragment-1 with Mn and ADP using magnetic resonance techniques, *J. Biol. Chem.*, 251, 1975, 1976.
10. Bagshaw, C. R. and Trentham, D. R., The reversibility of adenosine triphosphate cleavage by myosin, *Biochem. J.*, 133, 323, 1973.
11. Bagshaw, C. R. and Trentham, D. R., The characterisation of myosin-product complexes and of product release steps during the magnesium ion-dependent adenosine triphosphatase reaction, *Biochem. J.*, 141, 331, 1974.
12. Bagshaw, C. R., Trentham, D. R., Wolcott, R. G., and Boyer, P. D., Oxygen exchange in the γ-phosphoryl group of bound ATP during Mg^{2+}-dependent adenosine triphosphatase activity of myosin, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 2592, 1975.
13. Barany, M., ATPase activity of myosin correlated with speed of muscle shortening, *J. Gen. Physiol.*, 50, 197, 1967.
14. Barany, M., Bailin, G., and Barany, K., Reaction of myosin with 1-fluoro-2,4-dinitrobenzene at low ionic strength, *J. Biol. Chem.*, 244, 648, 1969.
15. Beinfeld, M. C. and Martonosi, A. N., Effect of F-actin upon the binding of ADP to myosin and its fragments, *J. Biol. Chem.*, 250, 7871, 1975.
16. Benson, S. W., *The Foundation of Chemical Kinetics,* McGraw-Hill, New York, 1960, 525.
17. Borejdo, J. and Putnam, S., Polarization of fluorescence from single skinned glycerinated rabbit Psoas fibers in rigor and relaxation, *Biochim. Biophys. Acta,* 459, 578, 1977.
18. Bremel, R. D., Myosin linked calcium regulation in vertebrate smooth muscle, *Nature* (London), 252, 405, 1974.
19. Bremel, R. D., Murray, J. M., and Weber, A., Manifestations of cooperative behavior in the regulated actin filament, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 267, 1972.
20. Bremel, R. D. and Weber, A., Cooperativity with actin filaments in vertebrate muscle, *Nature* (London), 328, 97, 1972.
21. Bremel, R. D. and Weber, A., Ca binding to rabbit skeletal myosin under physiological conditions, *Biochim. Biophys. Acta,* 376, 366, 1975.
22. Burke, M., Reisler, E., and Harrington, W. F., Effect of bridging the two essential thios of myosin on its spectral and actin-binding properties, *Biochemistry,* 15, 1923, 1976.
23. Burke, M., Reisler, E., Himmelfarb, S., and Harrington, W. F., Convergence of activation by actin and SH-1 modification at physiological ionic strength, *J. Biol. Chem.*, 249, 6361, 1975.
24. Carlson, F. D., Structural fluctuations in the steady state of muscle contraction, *Biophys. J.*, 15, 633, 1975.
25. Carlson, F. D. and Fraser, A., Dynamics of F-actin and F-actin complexes, *J. Mol. Biol.*, 89, 273, 1974.
26. Chacko, S., Conti, M. A., and Adelstein, R. S., Effect of phosphorylation of smooth muscle myosin on actin activation and Ca regulation, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 129, 1977.
27. Chantler, P. D. and Gratzer, W. B., Interaction of actin monomers with myosin heads and other muscle proteins, *Biochemistry,* 15, 2219, 1976.
28. Chen, Y. and Hill, T. L., Analysis of a simple prototypal muscle model near to and far from equilibrium, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 1982, 1974.

29. Chock, S. P. and Eisenberg, E., Heavy meromyosin Mg-ATPase: presteady-state and steady state H^+ release, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 4915, 1974.
30. Chock, S. P. and Eisenberg, E., Binding of ATP to myosin subfragment-1. Relation of fluorescence change to the binding and hydrolysis of ATP, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 36, 830, 1977.
31. Chock, S. P., Chock, P. B., and Eisenberg, E., Pre steady state kinetic evidence for a cyclic interaction of myosin subfragment-one with actin during the hydrolysis of adenosine 5'-triphosphate, *Biochemistry*, 15, 3244, 1976.
32. Cohen, C., Caspar, D. L. D., Johnson, J. P. , Nauss, K., Margossian, S. S., and Parry, D. A. D., Tropomyosin-troponin assembly, *Cold Spring Harbor Symp. Quant. Biol.*, 36, 287, 1972.
33. Cooke, R. and Franks, K. E., Production of force by single-headed myosin, *Biophys. J.*, 17, 41a, 1977.
34. Crooks, R. and Cooke, R., Tension generation by threads of contractile proteins, *J. Gen. Physiol.*, 69, 37, 1977.
35. Crothers, D. M. and Metzger, H., The influence of polyvalency on the binding properties of antibodies, *Immunochemistry*, 9, 341, 1972.
36. d'Albis, A. and Gratzer, W. B., Chemical cross linking of myosin, *J. Biol. Chem.*, 251, 2825, 1976.
37. Daniel, J. L. and Adelstein, R. S., Isolation and properties of platelet myosin light chain kinase, *Biochemistry*, 15, 2370, 1976.
38. Daniel, J. L. and Hartshorne, D. I., Reaction of myosin with NEM in the presence of ADP, *Biochim. Biophys. Acta*, 347, 151, 1974.
39. Dos Remedios, C., Yount, R. and Morales, M. F., Individual states in the cycle of muscle contraction, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 2542, 1972.
40. Duke, J., Takashi, K., Ue, K., and Morales, M. F., Reciprocal reactivities of specific thiols when actin bindings to myosin, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 302, 1976.
41. Eaton, B. L., Kominz, P. R., and Eisenberg, E., Correlation between the inhibition of acto HMM ATPase and the binding of tropomyosin to F-actin, *Biochemistry*, 14, 2718, 1975.
41a. Ebashi, S., *Essays Biochem.*, 10, 1, 1974.
42. Ebashi, S., Mikawa, T., Hirata, M., Toyo-Oka, T., and Nonomura, Y., Symposium on excitation contraction coupling in smooth muscle, *27th Int. Congr. Physiol. Sci.*, in press.
43. Ebashi, S., Toyo-Oko, T., and Nonomura, Y., Gizzard troponin, *J. Biochem.* (Tokyo), 78, 859, 1975.
44. Eccleston, J. F., Greeves, M. A., Trentham, D. R., Bagshaw, C. R., and Mrwa, J., Binding and cleavage of ATP in the myosin and actomyosin ATPase mechanisms, in *Molecular Basis of Motility*, Heilmeyer, L. M. G., Jr., Ruegg, J. C., and Wieland, Th., Eds., Springer-Verlag, Berlin, 1976, 42.
45. Eisenberg, E., Dobkin, L., and Keilley, W. W., Heavy meromyosin: evidence for a refractory state unable to bind to actin in the presence of ATP, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 667, 1972.
46. Eisenberg, E. and Hill, T. L., A cross bridge model of muscle contraction, *Prog. Biophys. Mol. Biol.*, in press.
47. Eisenberg, E., and Keilley, W. W., Native tropomyosin: effect on the interaction of actin with heavy meromyosin and subfragment-1, *Biochem. Biophys. Res. Commun.*, 40, 50, 1970.
48. Eisenberg, E. and Kielley, W. W., Evidence for a refractory state of heavy meromyosin and subfragment-1 unable to bind to actin in the presence of ATP, *Cold Spring Harbor Symp. Quant. Biol.*, 36, 145, 1972.
49. Eisenberg, E. and Moos, C., Actin activation of heavy meromyosin adenosine triphosphatase. Dependence on adenosine triphosphate and actin concentrations, *J. Biol. Chem.*, 245, 2451, 1970.
50. Elzinga, M., The reactive sulfhydryl groups SH1 and SH2 are close together in the primary structure of myosin, *Biophys. J.*, 17, 36a, 1977.
51. Finlayson, B. and Taylor, E. W., Hydrolysis of nucleoside triphosphates by myosin during the transient state, *Biochemistry*, 8, 802, 1969.
52. Frank, G. and Weeds, A. G., Amino acid of the alkali light chains of rabbit skeletal muscle myosin, *Eur. J. Biochem.*, 44, 317, 1974.
53. Gillis, J. M. and O'Brien, E. J., Effect of Ca on reconstituted thin filaments, *J. Mol. Biol.*, 99, 445, 1975.
54. Goody, R. S., Hofman, W., and Mannherz, H. G., The binding constant of ATP to myosin S1 fragment, *Eur. J. Biochem.*, 78, 317, 1977.
55. Goody, R. S., Holmes, K. C., Mannherz, H. G., Barrington-Leigh, J., and Rosenbaum, G., Cross bridge conformation as revealed by X-ray diffraction studies of insect flight muscles with ATP analogues, *Biophys. J.*, 15, 687, 1975.
56. Gordon, A. M., Huxley, A. F., and Julian, F. J., The variation in isometric tension with sarcomere length in vertebrate muscle fiber, *J. Physiol.*, (London), 185, 170, 1966.
57. Gorecka, A., Aksoy, M. O., and Hartshorne, D. J., Effect of phosphorylation of gizzard myosin on actin activation, *Biochem. Biophys. Res. Commun.*, 71, 325, 1976.
58. Gratzer, W. B. and Lowey, S., Effect of substrate on the conformation of myosin, *J. Biol. Chem.*, 244, 22, 1969.

59. Green, L. E. and Yount, R. G., Reaction of cardiac myosin with purine disulfide analogues of ATP, *J. Biol. Chem.*, 252, 1673, 1977.
60. Green, L. E. and Yount, R. G., Kinetics of inactivation and binding of adenylyl imido-diphosphate, *J. Biol. Chem.*, 252, 1681, 1977.
61. Gutfreund, H., Kinetic analysis of the properties and reactions of enzymes, *Prog. Biophys. Mol. Biol.*, 29, 161, 1975.
62. Hanson, J., Lednev, V., O'Brien, E. J., and Bennett, P. M., Structure of the actin containing filaments in vertebrate skeletal muscle, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 311, 1972.
63. Harrington, W. F., Reisler, E., and Burke, M., Activation mechanism of ATP cleavage in muscle, *J. Supramol. Struct.*, 3, 112, 1975.
64. Haselgrove, J. C., X-Ray evidence for a conformational change in the actin-containing filaments of vertebrate striated muscle, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 341, 1972.
65. Haselgrove, J. C., X-Ray evidence for conformational changes in the myosin filaments of vertebrate striated muscle, *J. Mol. Biol.*, 92, 113, 1975.
66. Haselgrove, J. C.,Structural features of the myosin filaments in vertebrate striated muscles, submitted.
67. Haselgrove, J. C. and Huxley, H. E., X-Ray evidence for rapid cross bridge movement and the sliding filament model in actively contracting skeletal muscle, *J. Mol. Biol.*, 77, 549, 1973.
68. Haselgrove, J. C., Stewart, M., and Huxley, H. E., (1976). Cross bridge movement during muscle contraction, *Nature* (London), 261, 606, 1976.
69. Highsmith, S., Interaction of the actin and nucleotide binding sites on myosin subfragment-1, *J. Biol. Chem.*, 251, 6170, 1976.
70. Highsmith, S., Effects of temperature and salts on myosin subfragment-1 and actin association, *Arch. Biochem. Biophys.*, 180, 404, 1977.
71. Highsmith, S., Association of heavy meromyosin and F-actin, *Biophys. J.*, 17, 40a, 1977.
72. Highsmith, S., Heavy meromyosin binds actin with negative cooperativity, *Biochemistry*, 17, 22, 1978.
73. Highsmith, S., Mendelson, R. A.,,and Morales, M. F., Affinity of myosin S-1 for F-actin, measured by time resolved fluorescence anisotropy, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 133, 1976.
74. Hill, T. L., Some statistical problems concerning linear macromolecules, *J. Polym. Sci.*, 231, 549, 1957.
75. Hill, T. L., Theoretical formalism for the sliding filament model of contraction of striated muscle, Part I, *Prog. Biophys. Mol. Biol.*, 28, 267, 1974.
76. Hill, T. L., Theoretical formalism for the sliding filament model of contraction of striated muscle, Part III, *Prog. Biophys. Mol. Biol.*, 29, 105, 1975.
77. Hill, T. L., *Free Energy Transduction in Biology*, Academic Press, New York, 1977.
78. Hitchcock, S. E., Regulation of muscle contraction. Binding of troponin and its components to actin and tropomyosin, *Eur. J. Biochem.*, 52, 255, 1975.
79. Hitchcock, S. E., Huxley, H. E., and Szent-Gyorgyi, A. G., Calcium sensitive binding of troponin to actin-tropomyosin: a two-site model for troponin action, *J. Mol. Biol.*, 80, 825, 1973.
80. Holt, J. C. and Lowey, S., Distribution of alkali light chains in myosin, *Biochemistry*, 16, 4398, 1977.
81. Hozumi, T. and Tawada, K., Kinetics of steady state ATPase activity and rigor complex formation of acto-HMM, *Biochim. Biophys. Acta*, 347, 469, 1974.
82. Hozimi, T. and Tawada, K., Temperature-dependent transitions of the myosin-product intermediate at 10°C in the MN(II)-ATP hydrolysis, *Biochim. Biophys. Acta*, 376, 1, 1975.
83. Huxley, A. F., Muscle structure and theories of contraction, *Prog. Biophys. Biophys Chem.*, 7, 255, 1957.
84. Huxley, H. E., Structural difference between resting and rigor muscles, *J. Mol. Biol.*, 37, 507, 1968.
85. Huxley, H. E., The mechanism of muscle contraction, *Science*, 164, 1356, 1969.
86. Huxley, H. E., Structural changes in the actin- and myosin-containing filaments during contraction, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 361, 1972.
87. Huxley, H. E., The relevance of studies on muscle to problems of cell motility, in *Cell Motility, Cold Spring Harbor Conferences on Cell Proliferation*, Vol. 3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1976, 115.
88. Huxley, H. E. and Brown, W., The low angle X-ray diagram of vertebrate striated muscle and its behavior during contraction and rigor, *J. Mol. Biol.*, 30, 385, 1967.
89. Huxley, H. E. and Hanson, J., Changes in the cross striations of muscle during contraction and stretch and their structural interpretation, *Nature* (London), 173, 973, 1954.
90. Huxley, A. F. and Niedergerke, R., Structural changes in muscle during contraction, *Nature* (London), 173, 971, 1954.
91. Huxley, A. F. and Simmons, R. M., Proposed mechanism of force generation in striated muscle, *Nature* (London), 233, 533, 1976.

92. Inoue, A., Shibata-Sekiya, K., and Tonomura, Y., Formation and decomposition of the myosin phosphate ADP complex, *J. Biochem.* (Tokyo) 71, 115, 1972.
93. Inoue, A., Shigekawa, M., and Tonomura, Y., Direct evidence for the two route mechanism of acto H-meromyosin ATPase reactions, *J. Biochem.*, (Tokyo), 74, 923, 1973.
94. Inoue, A. and Tonomura, Y., Kinetic properties of the myosin-phosphate-ADP complex, *J. Biochem.*, (Tokyo), 73, 555, 1973.
95. Inoue, A. and Tonomura, Y., Binding of adenosine and triphosphates to myosin during the hydrolysis of adenosine triphosphate, *J. Biochem.* (Tokyo), 76, 755, 1974.
96. Inoue, A. and Tonomura, Y., Separation of subfragment 1 of HMM into two equi molar fractions with and without formation of the reactive enzyme-phosphate ADP complex, *J. Biochem.* (Tokyo), 79, 419, 1976.
97. Inoue, A. and Tonomura, Y., Separation of the two fractions of subfragment 1 of myosin by affinity chromatography on immobilized F-actin, *J. Biochem.*, (Tokyo), 80, 1359, 1976.
98. Jencks, W. P., Binding energy specificity and enzyme catalysis, in *Advances in Enzymology,* Vol. 43, Meister, A., Ed., John Wiley & Sons, New York, 1975, 219.
99. Johnson, K. A. and Taylor, E. W., The intermediate states of subfragment 1 and acto-subfragment 1 ATPase — a reevaluation of the mechanism, *Biochemistry,* 17, 3432, 1978.
100. Johnson, K. A. and Taylor, E. W.,Comparison of the kinetic behavior of sub-fragment 1 and heavy meromyosin, in preparation.
101. Johnson, K. A., Trybus, K. M., and Taylor, E. W., Kinetics of myosin fluorescence changes, *Biophys. J.*, 21, 44a, 1978.
102. Kanazawa, T. and Tonomura, Y., Pre steady state of the myosin-adenosine triphosphate system. I. Rapid initial liberation of inorganic phosphate, *J. Biochem.*, (Tokyo), 57, 604, 1965.
103. Katz, A. M., Influence of tropomyosin on the reactions of actomyosin at low ionic strength, *J. Biol. Chem.*, 239, 3304, 1964.
104. Kendrick-Jones, J., Szentkiralyi, E., and Szent-Gyorgyi, A. G., Regulatory light chains in myosin, *J. Mol. Biol.*, 104, 747, 1976.
105. Kielley, B. and Martonosi, A., The binding of ADP to myosin, *Biochim. Biophys. Acta,* 172, 158, 1969.
106. Kodama, T. and Woledge, R. C., Calorimetric studies of the interaction of myosin with ADP, *J. Biol. Chem.*, 251, 7499, 1976.
107. Koretz, J. F., Hunt, T., and Taylor, E. W., Studies on the mechanism of myosin and actomyosin ATPase, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 179, 1972.
108. Koretz, J. F. and Taylor, E. W., Transient state kinetic studies of proton liberation by myosin and subfragment 1, *J. Biol. Chem.* , 250, 6344, 1975.
109. Kretzinger, R. H., Calcium binding proteins, *Annu. Rev. Biochem.*, 45, 239, 1976.
110. Kuhn, H. J., Reversible transformation of mechanical work into chemical free energy, in *Insect Flight Muscle,* Tregear, R. T., Ed., North-Holland, Amsterdam, 1977, 307.
111. Lehman, W., Ca ion dependent myosin from decapod crustacean muscles, *Biochem. J.*, 163, 291, 1977.
112. Lehman, W. and Szent-Gyorgyi, A. G., Distribution of actin control and myosin control in the animal kingdom, *J. Gen. Physiol.*, 66, 1, 1975.
113. Levy, H. M., Sharon, N., Ryan, E. M., and Koshland, D. E., Jr., Effects of temperature on the rate of hydrolysis of adenosine triphosphate and inosine triphosphate by myosin with and without modifiers, *Biochim. Biophys. Acta,* 56, 118, 1962.
114. Lowey, S. and Luck, S. M., Equilibrium binding of adenosine diphosphate to myosin, *Biochemistry,* 8, 3195, 1969.
115. Lowey, S. and Risby, D., Light chains of fast and slow muscle myosins, *Nature* (London), 234, 81, 1971.
116. Lymn, R. W., Effect of modifier on three-step enzymic process: general rate equation, *J. Theor. Biol.* (London), 43, 305, 1974.
117. Lymn, R. W., Actin activation of the myosin ATPase: a kinetic analysis, *J. Theor. Biol.*, (London), 43, 313, 1974.
118. Lymn, R. W., Low angle X-ray diagram from skeletal muscle, the effect of AMPPNP, *J. Mol. Biol.*, 99, 567, 1975.
119. Lymn, R. W. and Cohen, G. H., Equatorial X-ray reflections and cross arm movement in skeletal muscle, *Nature* (London), 258, 770, 1975.
120. Lymn, R. W. and Huxley, H. E., X-Ray diagrams from skeletal muscle in the presence of ATP analogues, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 449, 1972.
121. Lymn, R. W. and Taylor, E. W., Transient state phosphate production in the hydrolysis of nucleotide triphosphates by myosin, *Biochemistry,* 9, 2975, 1970.
122. Lymn, R. W. and Taylor, E. W., Mechanism of adenosine triphosphate hydrolysis by actomyosin, *Biochemistry,* 10, 4617, 1971.

123. Mannherz, H. G. and Goody, R. S., Proteins of contractile systems, *Annu. Rev. Biochem.*, 45, 427, 1976.
124. Mannherz, H. G., Schenck, H., and Goody, R. S., Synthesis of ATP from ADP and inorganic phosphate at the myosin-subfragment 1 active site, *Eur. J. Biochem.*, 48, 287, 1974.
125. Margossian, S. S. and Cohen, C., Troponin subunit interactions, *J. Mol. Biol.*, 81, 409, 1973.
126. Margossian, S. S. and Lowey, S., Substructure of the myosin molecule. IV. Interaction of myosin and its subfragment with adenosine triphosphate and F-actin, *J. Mol. Biol.*, 74, 313, 1973.
127. Margossian, S. S. and Lowey, S., Interaction of myosin subfragments with actin, *Biochemistry*, 17, 5431, 1978.
128. Margossian, S. S., Lowey, S., and Barshop, B., Effect of DTNB light chain on the interaction of vertebrate skeletal myosin with actin, *Nature* (London), 258, 163, 1975.
129. Marsh, D. J., deBruin, S. H., and Gratzer, W. B., HMM-ADP binding equilibrium from proton release measurements, *Biochemistry*, 16, 1738, 1977.
130. Marston, S. A., The association of subfragment 1 and HMM with actin, *Biochemistry*, submitted.
131. Marston, S. A., Complex kinetics of actin-subfragment 1 ATPase at low temperature, *FEBS Lett.*, 92, 147, 1978.
132. Marston, S. B., Rodger, C. D., and Tregear, R. T., Changes in muscle crossbridges when β,γ-imido-ATP binds to myosin, *J. Mol. Biol.*, 104, 263, 1976.
133. Marston, S. and Weber, A., Dissociation constant of actin-subfragment 1 complex, *Biochemistry*, 14, 3868, 1975.
134. Marston, S. A. and Taylor, E. W., Comparative studies on the intermediate states of acto-subfragment 1 ATPase, *J. Mol. Biol.*, submitted.
135. Marston, S. A. and Taylor, E. W., Comparative studies on the intermediate states of subfragment 1 ATPase of fast and slow striated and smooth muscles, *J. Mol. Biol.*, submitted.
136. Martonosi, A. and Malik, M. N., Kinetics of formation and dissociation of H-meromyosin-ADP complex, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 184, 1972.
137. McLachlen, A. D. and Stewart, M., The 14-fold periodicity in α-tropomyosin and the interaction with actin, *J. Mol. Biol.*, 103, 271, 1976.
138. Mendelson, R. A. and Cheung, P. H., Muscle crossbridges. Absence of direct effect of Ca on movement from thick filaments, *Science*, 194, 190, 1976.
139. Mendelson, R. A., Morales, M. F., and Botts, J., Segmental flexibility of the S-1 moiety of myosin, *Biochemistry*, 12, 2250, 1973.
140. Mikawa, T., Toyo-Oka, T., Nonomura, Y., and Ebashi, S., Essential factor of gizzard troponin fraction — a new type of regulatory protein, *J. Biochem.* (Tokyo), 81, 273, 1977.
141. Miller, A. and Tregear, R. T., Structure of insect fibrillar flight muscle in the presence and absence of ATP, *J. Mol. Biol.*, 70, 85, 1972.
142. Millman, B. M. and Bennett, P. M., Structure of the cross striated adductor muscle of the scallop, *J. Mol. Biol.*, 103, 439, 1976.
143. Moore, P. B., Huxley, H. E., and De Rosier, D. J., Three dimensional reconstruction of F-actin, thin filaments and decorated thin filaments, *J. Mol. Biol.*, 50, 279, 1970.
144. Moos, C. Actin activation of heavy meromyosin and subfragment-1 ATPases, *Cold Spring Harbor Symp. Quant. Biol.*, 36, 137, 1972.
145. Morgan, M., Perry, S. V., and Ottaway, J., Myosin light chain phosphatase, *Biochem. J.*, 157, 687, 1976.
146. Morimoto, K. and Harrington, W. F., Substructure of thick filament of vertebrate striated muscle, *J. Mol. Biol.*, 83, 83, 1974.
147. Morimoto, K. and Harrington, W. F., Evidence for structural changes in vertebrate thick filaments induced by calcium, *J. Mol. Biol.*, 88, 693, 1964.
148. Morita, F., Interaction of heavy meromyosin with substrate. II. Rate of the formation of ATP-induced ultraviolet difference spectrum of heavy meromyosin measured by stopped-flow method, *Biochim. Biophys. Acta*, 172, 319, 1969.
149. Morita, F., Interaction of HMM with substrate. V. Heterogeneity in the binding of ATP, *J. Biochem.*, (Tokyo), 69, 517, 1971.
150. Morita, F. and Ishigami, F., Temperature dependence of the decay of the UV absorption difference spectrum of HMM induced by ATP and ITP, *J. Biochem.* (Tokyo), 81, 305, 1977.
151. Mulhern, S. A. and Eisenberg, E., Further studies on interaction of actin with HMM and subfragment 1 in presence of ATP, *Biochemistry*, 15, 5702, 1976.
152. Mulhern, S., Eisenberg, E., and Keilley, W. W., Interaction of actin with NEM modified HMM in presence and absence of ATP, *Biochemistry*, 14, 3863, 1975.
153. Murphy, A. J., Circular dichroism of adenine and 6-mercaptopurine nucleotide complexes of HMM, *Arch. Biochem. Biophys.*, 163, 290, 1974.
154. Nauss, K. M., Kitagawa, S., and Gergely, J., Pyrophosphate binding to an α adenosine triphosphate activity of myosin and its proteolytic fragments, *J. Biol. Chem.*, 244, 755, 1969.

155. Nihei, T., Mendelson, A., and Botts, J., Use of fluorescence polarization to observe changes in attitude of S-1 moieties in muscle fibers, *Biophys. J.*, 14, 236, 1974.
156. Nihei, T., Mendelson, R. A., and Botts, J., The site of force generation in muscle contraction as deduced from fluorescence polarization studies, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 274, 1974.
157. O'Brien, E. J., Gillis, J. M., and Couch, J., Symmetry and molecular arrangement of paracrystals of reconstituted thin filaments, *J. Mol. Biol.*, 99, 461, 1975.
158. O'Brien, E. J., Morris, E. P., and Rowley, D. A., Structure of two types of actin plus tropomyosin paracrystals, Eur. Mol. Biol. Org. Alpbach Symp. Abstr., 1977.
159. Offer, G. and Elliott, A., Can a myosin molecule bind to two actin filaments? *Nature* (London), 271, 328, 1978.
160. Oosawa, F., Fuyime, S., Ishiwata, S., and Mihashi, K., Dynamic property of F-actin and thin filament, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 277, 1972.
161. Ohnishi, S., Maruyana, K., and Ebashi, S., Calcium induced conformational changes and mutual interactions of troponin components as studied by spin labelling, *J. Biochem.*, (Tokyo), 78, 73, 1975.
162. Parker, L., Pyun, H. Y., and Hartshorne, D. J., The inhibition of the adenosine triphosphatase activity of subfragment 1-actin complex by troponin B plus tropomyosin and troponin B, *Biochim. Biophys. Acta*, 223, 453, 1970.
163. Parry, D. A. D. and Squire, J. M., Structural role of tropomyosin in muscle regulation: analysis of the X-ray diffraction patterns from relaxed and contracting muscles, *J. Mol. Biol.*, 75, 33, 1973.
164. Paulsen, G., ^{32}P phosphate incorporation into ATP during ATP hydrolysis and its dependence on interaction of actin and myosin, *Eur. J. Biochem.*, 61, 77, 1976.
165. Peller, L., Segmental flexibility of the myosin molecule, *J. Supramol. Struct.*, 3, 169, 1975.
166. Pemrick, S. and Weber, A., Mechanism of inhibition of relaxation by *N*-ethylmaleimide, *Biochemistry*, 15, 5193, 1976.
167. Perry, S. V., Cole, H. A., Head, J. F., and Wilson, F. J., Location and mode of action of the inhibitory protein component of the troponin complex, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 251, 1972.
168. Pires, E., Perry, S. V., and Thomas, M. A. W., Myosin light chain kinase, *FEBS Lett.*, 41, 292, 1974.
169. Pollard, T. D. and Weihing, R. R., Actin and myosin and cell movement, *CRC Crit. Rev. Biochem.*, 2, 1, 1974.
170. Pope, B. J., Wagner, P. D., and Weeds, A. G., Heterogeneity of myosin heavy chains in subfragment 1 isozymes from rabbit skeletal myosin, *J. Mol. Biol.*, 109, 470, 1977.
171. Potter, J. D. and Gergely, J., Troponin, tropomyosin and actin interactions in the Ca regulation of muscle contraction, *Biochemistry*, 13, 2697, 1974.
172. Poulsen, F. R. and Lowy, J., X-ray studies on the dimensions of myosin projections in frog striated muscle, in *Contractile Systems in Non-Muscle Tissues*, Perry, S. V., Margreth, A., and Adelstein, R. S., Eds., North-Holland, Amsterdam, 1976, 342.
173. Reedy, M. K., Holmes, K. C. and Tregear, R. T., Induced changes in orientation of the cross bridges of glycerinated insect flight muscle, *Nature* (London), 207, 1276, 1965.
174. Reisler, E., Burke, M., and Harrington, W. F., Cooperative role of two sulfhydryl groups in myosin ATPase, *Biochemistry*, 13, 2014, 1974.
175. Reisler, E., Burke, M., Himmelfarb, S., and Harrington, W. F., Spacial proximity of two essential sulfhydryl groups of myosin, *Biochemistry*, 13, 3837, 1974.
176. Rosing, J. and Slater, E. C., The value of $\Delta G°$ for the hydrolysis of ATP, *Biochim. Biophys. Acta*, 267, 275, 1972.
177. Sarkar, S., Sreter, R., and Gergely, J., Light chains of myosins of white, red, and cardiac muscles, *Proc. Natl. Acad. Sci. U.S.A.*, 68, 946, 1971.
178. Schaub, M. C., Watterson, J. G., and Waser, P. G., Radioactive labeling of specific thiolgroups as influenced by ligand binding, *Hoppe-Seyler's Z. Physiol. Chem.*, 356, 325, 1975.
179. Schaub, M. C., Watterson, J. G., and Waser, P. G., Evidence for head-head interactions in myosin from cardiac and skeletal muscles, *Basic Res. Cardiol.*, 72, 124, 1977.
180. Schliselfeld, L. H., Steady state studies of actin activated ATPase of myosin, *Biochim. Biophys. Acta*, 445, 234, 1977.
181. Schliselfeld, L. A. and Barany, M., The binding of adenosine triphosphate to myosin, *Biochemistry*, 7, 3206, 1968.
182. Seidel, J., Effects of actin on the electron spin resonance of spin labeled myosin, *Arch. Biochem. Biophys.*, 157, 588, 1973.
183. Seidel, J. C., Similar effects of enzymic activity due to chemical modification of either of two sulfhydryl groups of myosin, *Biochim. Biophys. Acta*, 180, 216, 1969.
184. Seidel, J. C., Effect of ionic conditions, temperature and chemical modification on fluorescence of myosin during steady state of ATP hydrolysis, *J. Biol. Chem.*, 250, 5681, 1975.
185. Seidel, J. C. and Gergely, J., Electron spin resonance of myosin spin labeled at the S-1 thio groups during hydrolysis of ATP, *Arch. Biochem. Biophys.*, 158, 853, 1973.

186. Sekine, T., Barnett, L. M., and Keilley, W. W., Active site of myosin ATPase. I. Localization of one of the sulfhydryl groups, *J. Biol. Chem.*, 237, 2769, 1962.
187. Shigekawa, M. and Tonomura, Y., Activation of actomyosin ATPase by troponin, *J. Biochem.*, (Tokyo), 71, 147, 1972.
188. Shibata-Sekiya, K., Reaction intermediates of H-meromyosin ATPase and ultraviolet difference spectrum of H-meromyosin induced by ATP, *J. Biochem.*, (Tokyo), 79, 621, 1976.
189. Shukla, K. K. and Levy, H. M., Mechanism of oxygen exchange in actin-activated hydrolysis of ATP by subfragment-1, *Biochemistry*, 16, 132, 1977.
190. Shukla, K. K. and Levy, H. M., Comparative studies of oxygen exchange catalyzed by myosin, HMM and subfragment 1, *Biochemistry*, 16, 5199, 1977.
191. Sleep, J. A.,Transient Kinetics of Myosin ATPase, Ph.D. thesis, King's College, University of London, 1975.
192. Sleep, J. A. and Boyer, P. D., Effect of actin on intermediate oxygen exchange of myosin, *Biochemistry*, 17, 5417, 1978.
193. Sleep, J. A. and Taylor, E. W., Intermediate states of actomyosin ATPase, *Biochemistry*, 15, 5813, 1976.
194. Sleep, J. A., Hackney, D. D., and Boyer, P. D., Characterization of phosphate oxygen exchange reactions catalyzed by myosin through measurement of the distribution of ^{18}O-labeled species, *J. Biol. Chem.*, 253, 5235, 1978.
195. Sleep, J. A. and Hutton, R. L., Actin mediated release of ATP from a myosin ATP complex, *Biochemistry*, 17, 5423, 1978.
196. Sleep, J. A., Trybus, K. M., Johnson, K. A., Taylor, E. W., Kinetic studies of normal and modified HMM and subfragment 1, *J. Mechanochem. Cell Motil.*, in press.
197. Sobieszek, A., Ca-linked phosphorylation of a light chain of vertebrate smooth muscle, *Eur. J. Biochem.*, 74, 477, 1977.
198. Sobieszek, A. and Bremel, R. D., Preparation and properties of vertebrate smooth muscle myofibrils and actomyosin, *Eur. J. Biochem.*, 55, 49, 1975.
199. Sobieszek, A. and Small, J. V., Myosin-linked Ca regulation in vertebrate smooth muscle, *J. Mol. Biol.*, 102, 75, 1976.
200. Squire, J. M., General model of myosin filament structure. III. Molecular packing arrangements in myosin filaments, *J. Mol. Biol.*, 77, 291, 1973.
201. Squire, J. M., Muscle filament structure and muscle contraction, *Ann. Rev. Biophys. Bioeng.*, 4, 137, 1975.
202. Starr, R. and Offer, G. W., Polarity of the myosin molecule, *J. Mol. Biol.*, 81, 17, 1973.
203. Stone, D. B., Interaction of actin with spin-labeled heavy meromyosin in the presence of nucleotides, *Biochemistry*, 12, 3672, 1973.
204. Sutoh, K. and Harrington, W. F., Cross-linking of myosin thick filaments, *Biochemistry*, 16, 2441, 1977.
205. Szent-Gyorgyi, A. G., Szentkiralyi, E. M., and Kendrick-Jones, J., The light chains of scallop myosin as regulatory subunits, *J. Mol. Biol.*, 74, 179, 1973.
206. Taniguchi, S. and Tawada, K., Separation of myosin subfragment 1 into two fractions one having the burst site and the other having the non-burst site, *J. Biochem.* (Tokyo), 80, 853, 1976.
207. Taylor, R. S. and Weeds, A. G., Transient-phase of ATP hydrolysis by myosin sub-fragment-1 isoenzymes, *FEBS Lett.*, 75, 55, 1977.
208. Taylor, E. W. and Marston, S. A., Comparison of myosin and actomyosin ATPase cycles of fast and slow muscles, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 36, 830, 1977.
209. Taylor, E. W., Transient phase of adenosine triphosphate hydrolysis by myosin, HMM and subfragment 1, *Biochemistry*, 16, 732, 1977.
210. Taylor, E. W., Lymn, R. W., and Moll, G., Myosin product complex and its effect on the steady state rate of nucleotide triphosphate hydrolysis, *Biochemistry*, 9, 2984, 1970.
211. Thomas, D. D., Seidel, J. C., and Gergely, J., Molecular motions and the mechanism of muscle contraction and its regulation, in *Contractile Systems in Non-muscle Tissues*, Perry, S. V., Mograth, A., and Adelstein, R. S., Eds., North-Holland, Amsterdam, 1976, 13.
212. Thomas, D. D., Seidel, J. C., Hyde, J. S., and Gergely, J., Motion of subfragment-1 in myosin and its supramolecular complexes, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 1729, 1975.
213. Thomas, D. D., Seidel, J. C., and Gergely, J., Rotational motions of F-actin in the submillisecond time range, Eur. Mol. Biol. Org. Alpbach Symp. Abstr., 1977.
214. Tonomura, Y. and Kitagawa, S., The initial phase of actomyosin-adenosine triphosphatase. II. Factors influencing activity, *Biochim. Biophys. Acta*, 40, 135, 1960.
215. Tonomura, Y. and Morita, F., Binding of pyrophosphate to myosin A and myosin B, *J. Biochem.*, (Tokyo), 46, 1367, 1959.
216. Tonomura, Y., Nakamura, H., Kinoshita, N., Onishi, H., and Shigekawa, M., The reaction mechanism of the myosin ATP system and a molecular mechanism of muscle contraction, *J. Biochem.*, (Tokyo), 66, 599, 1969.

217. Tonomura, Y. and Inoue, E. A., The substructure of myosin and the reaction mechanism of its adenosine triphosphatase, *Mol. Cell. Biochem.*, 5, 127, 1974.
218. Tonomura, Y. and Inoue, A., Energy transducing mechanisms in muscle MTP, in *International Review of Science, Series One, III. Energy Transducing Mechanisms*, Racker, E., Ed., Butterworths, London, 1975, 121.
219. Trentham, D. R., personal communication, 1977.
220. Trentham, D. R., Bardsley, R. G., Eccleston, J. F., and Weeds, A. G., Elementary processes of the magnesium ion-dependent adenosine triphosphatase activity of heavy meromyosin, *Biochem. J.*, 126, 635, 1972.
221. Trentham, D. R., Eccleston, J. F., and Bagshaw, C. R., Kinetic analysis of ATPase mechanisms, *Q. Rev. Biophys.*, 9, 217, 1976.
222. Vilbert, P. J., Haselgrove, J. C., Lowy, J., and Poulson, F. R., Structural changes in actin-containing filaments of muscle, *J. Mol. Biol.*, 71, 757, 1972.
223. Wagner, P. D., Fractionation of heavy meromyosin by affinity chromatography, *FEBS Lett.*, 81, 81, 1977.
224. Wagner, P. D. and Weeds, A. G., Recombination of hybridization of light and heavy chains of subfragment 1 preparations, *J. Mol. Biol.*, 109, 455, 1977.
225. Wagner, P. D. and Yount, R. G., Stoichiometry of labeling of myosin's proteolytic fragments by a purine disulfide analog of adenosine triphosphate, *Biochemistry*, 14, 1900, 1975.
226. Wagner, P. D. and Yount, R. G., Purine disulfide analogue-reaction at a binding site other than the active site, *Biochemistry*, 14, 5156, 1975.
227. Wagner, P. D. and Yount, R. G., Subunit location of sulfhydryl groups of myosin labeled with a purine disulfide analog of adenosine triphosphate, *Biochemistry*, 14, 1908, 1975.
228. Wagner, P. D. and Yount, R. G., Modification of alkali light chains of skeletal myosin inhibits actin binding and ATP cleavage, *J. Biol. Chem.*, 251, 5424, 1976.
229. Wakabayashi, T., Huxley, H. E., Amos, L. A., and Klug, A., Three dimensional image reconstruction of the actin-tropomyosin and actin-tropomyosin-troponin I complexes, *J. Mol. Biol.*, 93, 477, 1975.
230. Watterson, J. G., Schaub, M. C., Locker, R., DiPierri, S., and Kutzer, M., Temperature induced conformation of intermediates in the hydrolylic cycle of myosin, *Eur. J. Biochem.*, 56, 79, 1975.
231. Webb, M. R., McDonald, G. G., and Trentham, D. R., personal communication, 1978.
232. Weber, A. and Hasselbach, W., Die Erhohung der rate der ATP-spaltung durch myosin—und aktomyosin—gele fur Geginn der Spaltung, *Biochim. Biophys. Acta*, 15, 237, 1954.
233. Weber, A. and Murray, J. M., Molecular control mechanisms in muscle contraction, *Physiol. Rev.*, 53, 612, 1973.
234. Weeds, A., Light chains of slow twitch muscle myosin, *Eur. J. Biochem.*, 66, 157, 1977.
235. Weeds, A. G., Hall, R., and Spurway, N. C., Characterization of myosin light chains from histochemically identified fibers of rabbit Psoas muscle, *FEBS Lett.*, 49, 320, 1975.
236. Weeds, A. G. and Taylor, R. S., Separation of subfragment-1 isoenzymes from rabbit skeletal muscle myosin, *Nature* (London), 257, 54, 1975.
237. Werber, M., Szent-Gyorgyi, A. G., and Fasman, G., Fluorescence studies on heavy meromyosin: substrate interaction, *Biochemistry*, 11, 2872, 1972.
238. Werber, M. M. and Oplatka, A., Evidence for a regulatory role of a myosin light chain, *Biochem. Biophys. Res. Commun.*, 57, 823, 1974.
239. White, H. D., Magnesium-ADP binding to acto-myosin S1 and acto-heavy meromyosin, *Biophys. J.*, 17, 40a, 1977.
240. White, H. D. and Taylor, E. W., Kinetic studies of the recombination of myosin intermediate states with regulated actin, unpublished observations, 1975.
241. White, H. D. and Taylor, E. W., Energetics and mechanism of actomyosin adenosine triphosphatase, *Biochemistry*, 15, 5818, 1976.
242. Winstanley, M. A., Trayer, H. R., and Trayer, I. P., Separation of subfragment 1 isoenzymes by differential actin binding, *FEBS Lett.*, 83, 141, 1977.
243. Wolcott, R. G. and Boyer, P. D., The reversal of the myosin and actomyosin ATPase reactions and the free energy of ATP binding to myosin, *Biochem. Biophys. Res. Commun.*, 57, 709, 1974.
244. Wray, J. S., Vibert, P. J., and Cohen, C., Cross bridge arrangement in liminlus, *J. Mol. Biol.*, 88, 343, 1974.
245. Wray, J. S., Vibert, P. J. and Cohen, C., Diversity of cross-bridge configurations in invertebrate muscles, *Nature* (London), 257, 561, 1975.
246. Yagi, N., Ito, M. H., Nakajima, H., Izumi, T. and Matsubara, I., Return of myosin heads to thick filaments after muscle contraction, *Science*, 197, 685, 1977.
247. Yazawa, M., Morita, F., and Yagi, K., Distinction between two moles of ADP binding to HMM in the presence of Mn, *J. Biochem.*, (Toyko), 74, 1107, 1973.

248. **Yoshida, M. and Morita, F.**, Binding of adenosine diphosphate to subfragment 1, *J. Biochem.* (Tokyo), 77, 983, 1975.
249. **Yoshida, M. and Morita, E. F.**, Separation of myosin subfragment 1 into fractions containing g_1 chain and g_3 chain by Sepharose-adipic acid hydrazido-ATP column chromatography, *J. Biochem.*, (Tokyo), 79, 1049, 1976.
250. **Young, J. H., McLick, J., and Korman, E. F.**, Psuedo rotation mechanism of ATP hydrolysis in muscle contraction, *Nature* (London), 249, 474, 1974.
251. **Yount, R. G., Ojala, D., and Babcock, D.**, Interaction of P-N-P and P-C-P analogs of adenosine triphosphate with heavy meromyosin, myosin and actomyosin, *Biochemistry*, 10, 2490, 1971.