

THE MOLECULAR BASIS OF CROSS-BRIDGE FUNCTION

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1. INTRODUCTION

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

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

2. CROSS BRIDGE THEORIES

2.1 The swinging cross-bridge

After the discovery of the sliding filaments the question naturally arose; what made them slide? AF Huxley⁹ argued that the source of the force must be independent elemental force generators since the force increases linearly with the degree of filament overlap. The myosin cross bridges were first visualised by electron microscopy^{10, 11} and subsequently shown both to be the site of the ATP-ase and also to be the motor elements producing force and movement between the filaments. Two conformations of the cross-bridge could be detected in insect flight muscle by low angle X-ray scattering and electron microscopy¹². The cross bridge attaches to the actin filament at about 45° in rigor and at right angles (90°) in the presence of ATP.

On the basis of the known structural data and their kinetic analysis Lymn and Taylor proposed the cross-bridge cycle^{13, 14} (Fig. 1). The cross bridge was thought work by a kind of rowing action. Initially it would bind to actin in a 90° conformation, go over to an angled (45°) conformation and then release the products of hydrolysis. The rebinding of Mg-ATP rapidly dissociates the actin-myosin; myosin then hydrolyzes ATP and forms a stable myosin-products complex; actin recombines with this complex and dissociates the products thereby forming the initial actin-myosin complex that isomerises to the rigor complex. Force is generated during the last step.

The actual rowing movement could be measured by physiological experiments on contracting muscle and was shown to be about 100Å¹⁵. Since the cross-bridge was an elongated structure, such a distance could be accommodated by a rotating or swinging cross-bridge model (Fig 1a). Studies of the cross-bridge movement were undertaken by time resolved studies of contracting frog muscle using low angle x-ray fibre diffraction^{16, 17}. These results are fully consistent with the swinging cross-bridge theory.

2.2 Swinging lever arm

Although the swinging cross bridge hypothesis of muscle contraction had become the textbook norm by the time of the Cold Spring Harbor Conference on Muscle in 1972 it proved remarkably difficult to catch a bridge *in flagranti delicto* (Cooke¹⁸).

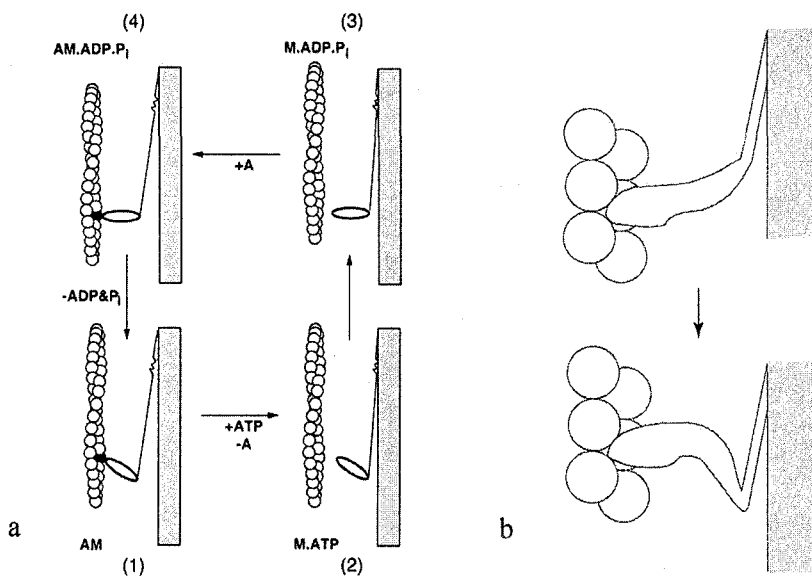


Figure 1. a The cross-bridge cycle from Lymn and Taylor ¹⁴. The actin filament is represented as a double helix of spheres. The myosin cross-bridge is connected to the myosin thick filament (shaded). The cross-bridge movement is depicted as a hinge on the surface of actin. b. The swinging lever arm as proposed by Cooke ¹⁸. The actin-myosin interface has a constant geometry; the movement takes place in a lever arm distal to the actin filament.

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

The atomic structures of actin and myosin provided new impetus. The crystal structure of the myosin subfragment 1 ¹⁹ showed the myosin cross-bridge to have an extended C-terminal tail which looked like a lever arm and, moreover, a lever arm which was in the correct orientation and position to function as a lever arm ²⁰. In the last years large numbers of independent experiments provide results, which are in excellent accord with the idea that the C-terminal tail functions as a lever arm and indeed provide evidence that it can move. Purified myosin cross bridges (S1) can be attached to a substrate and used to transport actin filaments in vitro in the presence of ATP. A study by Spudich *et al* ²¹ showed that the speed of actin transport in motility assays was proportional to the length of the lever arm. This experiment has

now been repeated a number of times on various myosins. Single molecule measurements come to similar conclusions. The bulk of the published data agree very well with the swinging lever arm model.

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

3. PROTEIN CRYSTALLOGRAPHY

3.1 Structure of Actin

Actin (thin filament) fibres are helical polymers of g-actin (globular-actin)²². The structure of g-actin was first solved by protein crystallography as a 1:1 complex with the enzyme DNase I²³. Orientated gels of f-actin yield X-ray fibre diagrams to about 6Å resolution. Holmes *et al* determined the orientation of the g-actin monomer that best accounted for the f-actin fibre diagram and thus arrive at first approximation to the atomic model of the actin filament²⁴. Since a conformational change is involved in going from g- to f-actin, the g-actin structure has to be deformed in some way to fit the f-actin diffraction pattern. The various attempts at generating the f-actin structure that have been published have chosen various methods of defining these free parameters and have ended up with related, but different, solutions to the problem²⁴⁻²⁶. The method of Lorenz *et al*²⁶ produces a very good fit to the fibre diffraction pattern but with a large number of free parameters and at the cost of poor stereochemistry. A new attempt to solve this problem has been made that is based on the sub-domain structure of actin and a small number of degrees of freedom²⁷. The g-actin structure from Otterbein *et al*²⁸ was used as the starting structure.

3.2 Structure of Myosin

The cross-bridges comprise a part of the myosin molecule, namely subfragment-1 of heavy meromyosin (S1). X-ray crystallography¹⁹ shows the chicken skeletal S1 to be tadpole-like in form (fig. 2), with an elongated head, containing a 7-stranded β -sheet and numerous associated α -helices forming a deep cleft. The cleft separates two parts of the molecule, which are referred to as the upper 50K, and lower 50K domains. The C-terminal tail, sometimes called the "neck", which also provides the connection to the thick filament, forms an extended α -helix that binds two calmodulin-like light chains. The ATP binding site consists of a "P-loop" motive flanked by switch 1 and switch 2 elements, as are found in the G-proteins.

By fitting the atomic structures of f-actin and S1 into three dimensional cryo-electron microscope reconstructions one arrives at an atomic model of the actin myosin complex²⁰ (fig. 3). In particular, this model establishes the spatial orientation of the S1 myosin fragment in the active complex. One finds that the cleft in myosin extends from the ATP binding site to the actin binding site and that

the opening and closing of this cleft is very likely to provide the communication between the ATP site and the actin binding site. The actin-binding site spans the upper and lower 50K domains. Furthermore, the very extended C-terminal α -helical neck of S1 is ideally placed to be a lever arm. The lever arm joins onto the bulk of the molecule via a small compact "converter domain"²⁹ which lies just distal to a broken α -helix containing two reactive thiol groups known as SH1 and SH2. Numerous experiments point to the putative "hinge" for the lever arm being in the SH1-SH2 region of the molecule (see³⁰ for review). The converter domain is the socket that carried the C-terminal helix (lever arm).

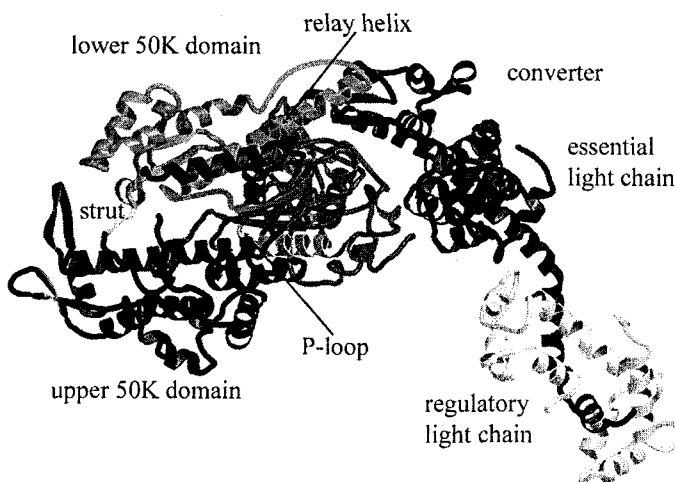


Figure 2 The structure of the myosin cross-bridge as a ribbon diagram¹⁹. The N-terminus is shown green and the nucleotide binding P-loop is shown yellow. The strut (yellow) connects the upper and lower 50K domains. Note the cleft separating the upper and lower 50K domains. The lower 50K domain is the primary actin-binding site. The upper and lower 50K domains are also connected by a disordered loop (not shown). The SH1 SH3 region lies underneath the right part of the relay helix. The C-terminal long helix (dark blue) carries two calmodulin-like light chains and joins on to the thick filament

3.3 Two main conformations of myosin have been discovered

According to the Lymn-Taylor scheme (fig. 1) the myosin cross bridge would be expected to have two discernible conformations: (1) when it first attaches to actin with the products of hydrolysis still bound with the lever at the beginning of the working stroke; and (2) at the end of the working stroke when the phosphate and ADP are released. This sequence is often referred to as the "power stroke". The end state is referred to as "rigor", since it is the state muscle enters on ATP depletion. It is also called "strong" because it binds to actin quite tightly. The initial state is called the "weak binding state" because of its low affinity for actin (see³¹), although it may be necessary to specify the pre-power stroke bound state as stereo-

specific weak binding.. We might anticipate that these two states of the myosin cross bridge might exist independently from actin and indeed protein crystallography shows this to be the case. The cross-bridge exists in two main conformations, the lever arm undergoes a 60° rotation between these two states, which have been identified as the beginning and end of the power stroke. The lever arm rotation is coupled with changes in the active site (the movement of the switch 2 element from closed to open) and to product release.

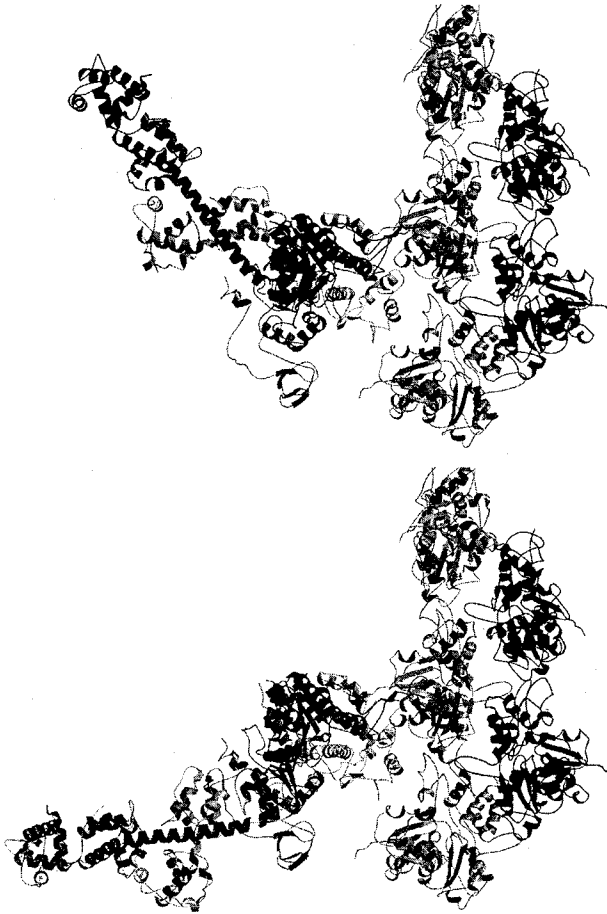


Figure 3 The pre (upper) and post (lower) power stroke states of the myosin cross bridge, as they would appear when attached to actin. The actin filament is shown on the right. These two states are referred to as CLOSED (upper) and OPEN (lower) because in the CLOSED state the switch 2 element has move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

3.3.1 The OPEN state (post power stroke)

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

3.3.2 The CLOSED state (pre-power stroke)

Rayment *et al* have extensively studied a crystalline fragment of the *dictyostelium* myosin II cross-bridge which has been truncated after residue 761 (equivalent to 781 in chicken skeletal sequence). The truncation eliminates the lever arm and the associated light chains but retains the converter domain. The crystal structures of the 761 construct have been determined with a number of ATP analogs, particularly ADP.BeF₃³² and ADP.vanadate³³. ADP.vanadate complexes are used as analogs of the transition state or possibly of the ADP.Pi state. While the ADP.BeF₃ state looks similar to rigor, the ADP.vanadate structure shows dramatic changes in shape of the S1 structure. There is a closing of the γ -phosphate binding pocket by moving switch 2 element about 5 Å. This induces large movements in the C-terminal region of the molecule. The converter domain rotates through 60°. This new state has been called "CLOSED" (because the nucleotide pocket is closed) or "transition state" because it seems to be produced by transition state analogues of ATP.

Dominguez *et al*³⁴ have solved the structures of chicken smooth-muscle myosin truncated at 791 (smooth muscle sequence) or at 820 (expressed in insect cells using the baculovirus vector). The shorter construct stops at the end of the converter domain and the longer construct encompasses the essential light-chain binding site that is, the first half of the lever arm. The structures of both constructs have been solved as complexes with ADP.vanadate and ADP.BeF₃. Both structures show the myosin cross-bridge in the CLOSED form with the converter domain in the rotated position very similar to that obtained in the two *Dictyostelium* constructs. The authors refer to this state as the "pre-power stroke state". There are no substantial differences between ADP.AlF₄ complexes and ADP.BeF₃ complexes showing that the nature of the ligand (either a transition state analog or an ATP analog) does not control the protein conformation very closely. Since the smooth-muscle crystals display extensive non-crystallographic symmetry, CLOSED has now been obtained 16 times in a large variety of different environments: there is little chance that it arises as an artefact of crystal packing.

The two states are depicted in fig. 3 as they would appear if they were full length cross bridges attached to actin. (The missing lever arm has been added in). The lever arm moves some 12nm along the actin helix axis between the two states.

The movement of the lever arm and the status of the nucleotide-binding pocket are tightly coupled: pocket closed, lever up (beginning); pocket open, lever down (end). Only the CLOSED form is an ATPase. This is an essential control to make the cross-bridge cycle efficient.

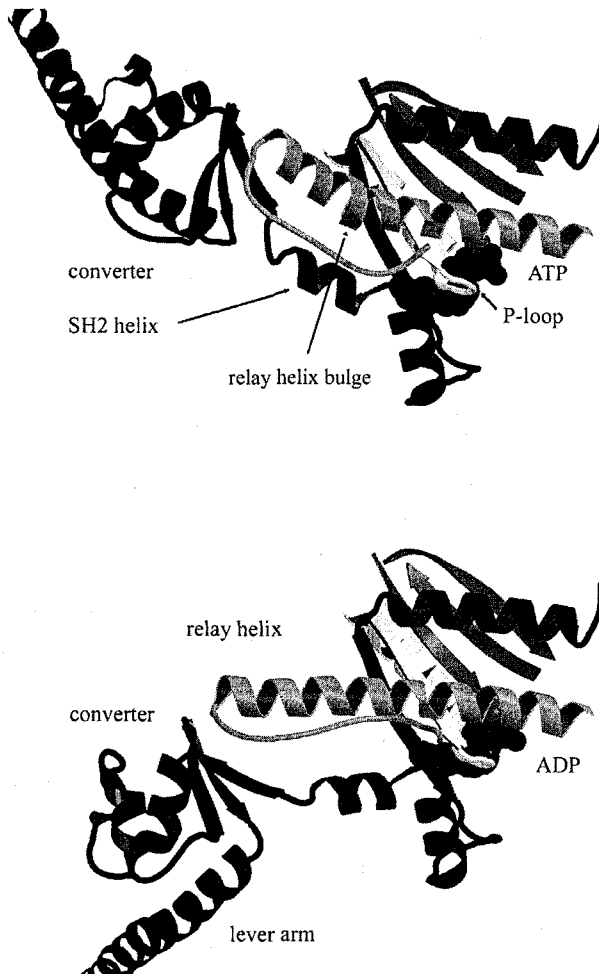


Figure 4 (Upper) Pre-power stroke and (lower) post-power stroke (near rigor) states. The removal of the bulge in the relay helix drives the rotation of the converter domain. The breaking of the relay helix appears to result from the helix being pressed against the β -sheet when the switch 2 element moves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

3.3.3 the relay helix bulge is the quintessence of muscle contraction

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

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

Whereas the structure of "decorated actin" an actin filament with a myosin cross-bridge bound to each actin in the rigor state has been extensively studied³⁷⁻⁴⁰ corresponding studies in the presence of ATP are difficult since the binding of ATP leads to rapid dissociation of the cross-bridges from actin. Time-resolved electron micrograph studies in fact show no bulk change of the cross bridge orientation on binding ATP before dissociation takes place⁴¹ whereby a reorientation of the lever arm would not have been detected at the resolution attainable. High-resolution electron micrographs of actin decorated with smooth muscle myosin, however, show a 30-35° rotation of the lever arm on binding ADP^{42, 43}. Although the main movement of the lever arm would be expected to be associated with phosphate release since this is a step associated with a large change in free energy, some fraction of the movement could arise from ADP binding and release. Moreover, this movement should be recoverable on adding ADP to actomyosin, which indeed it is. Although the effect has only been found in smooth muscle myosins this experiment was important in providing the first direct demonstration of a nucleotide-induced lever-arm swing.

5. MYOSIN V, A PROCESSION MYOSIN

Myosin II and myosin V have rather similar structures except that the lever arm on myosin V is long enough to binds 6 calmodulin light chains rather than 2 (myosin II cross bridges are about 16 nm long, myosin V cross bridges about 31 nm long). Whereas the two heads of myosin II act independently the two heads of

myosin V bind conjointly to the actin filament to produce a processive linear motion along actin by a hand over hand mechanism. The myosin V lever arms are long enough to allow two-headed binding that spans the actin helical repeat (approximately 36 nm). Electron microscopy has shown that while working, myosin V spans the actin helical repeat⁴⁴. The heads are mostly 13 actin subunits apart. Typically the lead head appears curved. The leading head may correspond to the beginning of the working stroke of the motor. Besides providing a graphical demonstration of the swinging lever arm myosin V offers the possibility of studying the elusive top-of-power-stroke state.

7. WEAK AND STRONG BINDING

The actin binding site straddles a cleft between two sub-domains, the upper and lower 50K domains.. Kinetic analysis shows that the initial binding of myosin to actin is "weak" and that weak binding isomerises to "strong"³¹. Strong binding drives the power stroke and affects ATP and ADP affinity. All myosin II crystal structures whether OPEN or CLOSED have an open 50K domain cleft and appear to be in the weak actin binding form. Since myosin V binds more tightly to actin it there is a chance that the myosin cross-bridge alone might be found in the strong binding form. X-ray crystallography has recently given the structure of the myosin V cross-bridge and indeed it appears to be in the strong binding form³⁵. Moreover, the interaction between the myosin cross-bridge and actin can be studied by cryo-electron microscopy⁴⁰. Together these studies explain the reciprocal linkage between actin binding and nucleotide binding: on strong binding to actin the 50k domain cleft closes leading to a movement of the switch 1 element out of the active site; the rebinding of ATP closes the active site by pulling in switch 1 thereby opening the cleft and weakening actin binding.

8. CONCLUSION

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

DISCUSSION

Ishiwata: The structural change of the motor domain of S1 obtained with and without Pi (inorganic phosphate) in the presence of ADP is very impressive. But, I think that the ADP-bound structure is that of the simple complex of ADP and S1. If this structural change is responsible for the power stroke, it means that the force is generated by the dissociation of Pi from the S1-ADP complex. I suppose that the structure of S1-ADP complex obtained after the Pi release step in the ATP hydrolysis-cycle is different from that of the S1-ADP complex.

Holmes: I wish I could answer your question, but I am a mere crystallographist so that I only construct a model based on the crystallographic structures available so far.

Huxley: Dr. Brenner has suggested that initial tension development by cross-bridges takes place by a different process from that involved in the sliding movement. The closure of the cleft in the 50K subunit of S1 could be the source of this initial force.



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