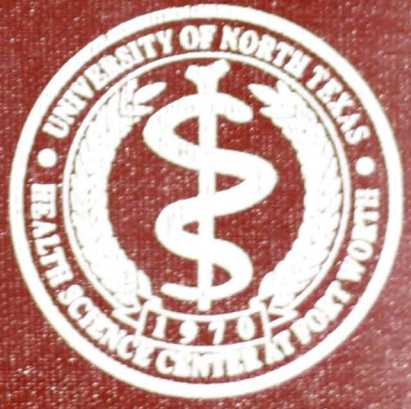
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Ushakov, Dmitriy S., Conformational transitions in myosin subfragment-1. Doctor of Philosophy (Biochemistry), June 2000, 72 pp., 7 tables, 19 illustrations, bibliography: 139 titles.

The contraction of muscles is driven by ATP-dependent interaction of actin and myosin filaments. It has been recently shown that the regulatory domain (RD) of smooth muscle myosin, containing both the regulatory and essential light chains, exists in different orientations depending on the nucleotide bound to the myosin ATPase site. However, this could not be detected in skeletal muscle myosin, and therefore it is still not known whether it is the RD or the change in the myosin motor domain (MD) that is responsible for the force production. To investigate this, we used chemical cross-linking to analyze the binding of myosin subfrgment-1 (S1) to F-actin in the presence of various adenine nucleotides. We found that ADP causes the reorientation of S1 with respect to F-actin, but only at physiological molar ratio of S1 to actin. The result can be simply explained by the two-state model of S1 binding to F-actin proposed earlier, in which S1 binds to one (state 1) or to two (state 2) actin monomers, depending on the saturation of the filaments with S1. This suggests that the change in the orientation of RD could be a mere consequence of the conformational change in the MD.

To investigate the changes in the RD further, we used a fluorescence anisotropy of an external fluorophore attached to a specific cysteine residue of the protein. To facilitate experiments, a tag of 6 histidines was genetically introduced at the C-terminus of LC1. The

recombinant LC1 was labeled with rhodamine at the cysteine 178 near the C-terminus, and exchanged into free S1 or in muscle fibers. The fluorescence anisotropy showed that the LC1 becomes more immobilized in the presence of ATP compared to the rigor state. The fact that ATP increases immobilization of LC1 suggests that the conformational changes take place in the RD of S1 during the ATP hydrolysis. The ordering of the LC1 could be due to the ATP-induced closure of the cleft between a small β-sheet on LC1 (Cys178-Met145) and a flexible loop on the catalytic domain (Arg18-Arg24).

From presented evidences, we conclude that the conformational transitions in both the MD and RD of S1 contribute to the power stroke.

CONFORMATIONAL TRANSITIONS IN MYOSIN

SUBFRAGMENT-1

Dmitriy S. Ushakov

APPROVED:

Major Professor

Committee Member

Committee Member

Committee Member

Committee Member

University Member

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CONFORMATIONAL TRANSITIONS IN MYOSIN SUBFRAGMENT-1

DISSERTATION

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences
University of North Texas
Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Dmitriy S. Ushakov, M.S.

Fort Worth, Texas
August 2000

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List of Abbreviations

5'-IAF	5'-Iodoacetamido-fluorescein
5'-IATR	5'-Iodoacetamido-tetramethyl-rhodamine
A	Actin
ADP	Adenosine diphosphate
AMP-PNP	Adenylylimidodiphosphate
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
DTT	Ditiothreitol
ε	Molar extinction coefficient
EDC	1-Ethyl-3-(3-dimethyl-aminopropyl)carbodiimide
EDTA	Ethylene-diamine-tetra-actetic acid
EGTA	Ethylene Glycol-bis(β-aminoethyl) ether
ELC	Essential light chain
F-Actin	Actin polymer
HC	Heavy chain of S1
IPTG	Isopropyl-β-D-thiogalactoside
LB	Luria-Bertani growth medium
LC1	Essential light chain 1
LC1m	Mutant LC1 containing Cys at position 2
LC3	Essential light chain 3
MD	Motor domain of S1
MR	Molar ratio of S1 to actin
NHS	N-hydroxysuccinimide
PBS	Physiologic buffer system
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PPi	Pyrophosphate
r	Anisotropy of fluorescence
RD	Regulatory domain of S1
RLC	Regulatory light chain
S1	Subfragment-1 of myosin
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TFP	Trifluoperazine
ρ	Rotational correlation time
τ	Excited state lifetime

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I thank my parents, family and friends for their permanent support.

CHAPTER ONE

INTRODUCTION

1. General theories of muscle contraction

The process of directional movement is a basis for life. Despite the variety of types of biological motility, all of them originate from just a few molecular mechanisms. In general, the motility of all eucaryotes is a result of the ATP-dependent interaction of two proteins, with the chemical energy of ATP hydrolysis being transformed into the mechanical energy of movement. The best understood mechanism of motility is based on the interaction of actin and myosin. It is this interaction that is responsible for the contraction of muscles and many other processes of cellular movement.

The research of striated muscles, which include the skeletal and cardiac muscles, began as early as in the 3rd century B.C., when the first theory of contraction appeared in Ancient Greece. The invention of the light microscope illuminated the ultrastructural characteristics of skeletal muscles, namely, that they have a striated pattern formed by alternating regions of different optical density, called anisotropic (A) and isotropic (I) bands. However, it was not until 1864 that Kühne (Kühne, 1864) discovered the protein myosin. And only in 1939 did Ljubimowa and Engelhardt show that the ATP was hydrolyzed by myosin and is the immediate energy source for muscle contraction (Engelhardt and

Ljubimowa, 1939). Straub later discovered that the "myosin" consisted actually of two proteins, myosin and actin (Straub, 1943). Nowadays, both actin and myosin are well characterized.

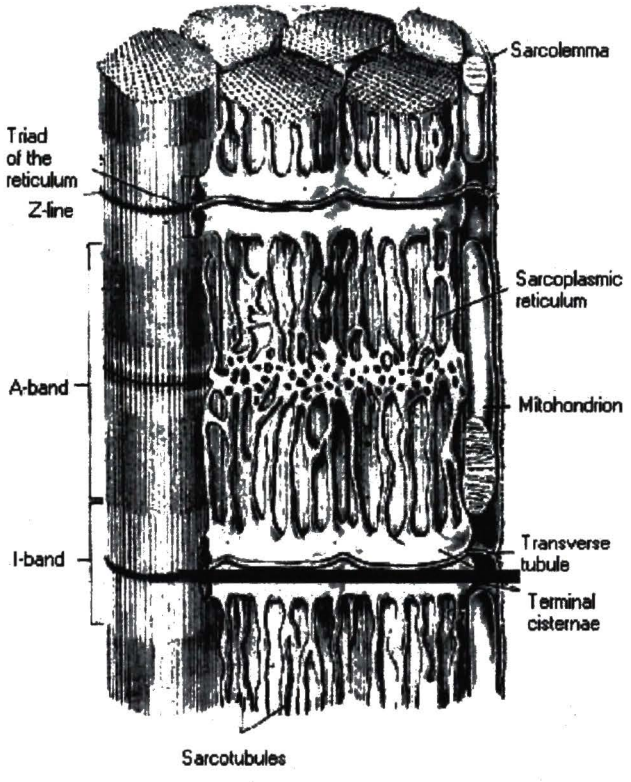

Figure 1: Ultrastructure of skeletal muscle fiber (Squire, 1981).

The striated muscles consist of long parallel bundles of muscle fibers up to 100 μm in diameter, which may span the entire length of the muscle. Muscle fibers are, in turn, composed of bundles of up to 2 μm-diameter myofibrils (Fig. 1). The repeating unit of myofibrils is a sarcomere, which is 2.5 to 3.0 μm long in relaxed muscle and 1/3 shorter in a contracted state. The sarcomere is bound by dark Z-lines at

the center of each I-band. The A-band is centered on the lighter H-zone, which, in turn, has a dark M-line in the center.

The first molecular theories of contraction appeared in the thirties and were based on principles of polymer science (Holmes, 1997). They suggested that there was a rubber-like shortening of myosin filaments brought about by altering state of ionization of the myosin. This notion was put aside by the seminal works of H.E. Huxley (Huxley and Hanson, 1954) and A.F. Huxley (Huxley and Niedegeke, 1954), who showed that sarcomeres contained two sets of filaments (thick and thin), which glided over each other without altering their length. Projections from the thick filaments, the myosin cross-bridges, were discovered by electron microscopy (Huxley 1957; Huxley 1958) and subsequently shown both to be the site of the ATPase 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 (Reedy *et al.*, 1965). The cross-bridge was thought to bind to thin filament in an initial (90°) conformation, go over to an angled (45°) conformation and then release (Huxley 1969; Lymn and Taylor 1971). For each cycle of activity one ATP molecule would be hydrolyzed. The actual movement could be measured by physiological experiments on contracting muscle and was shown to be about 80-100Å (Huxley and Simmons 1971). Since the cross-bridge was known to be an elongated structure, a rotating or swinging cross-bridge model could accommodate such a distance. Recently, the understanding

of actomyosin system has been greatly advanced by the successful crystallization of both actin and S1 of myosin.

Actin is a 42 kDa globular protein with an asymmetric structure. The X-ray crystallography of actin in a 1:1 complex with pancreatic DNAse I showed that it consists of two domains separated by a deep cleft, with each domain having two clearly distinct subdomains (Kabsch *et al.*, 1990). The ATP or ADP binds in a cleft between two domains. Actin polymerizes at physiological ionic strength and forms oriented helical filaments 50-100 Å in diameter and 1 μm in length, which have 13 molecules in 6 turns repeating every 360Å. During polymerization one molecule of ATP is hydrolyzed per actin monomer. These filaments form the backbone of the thin filaments of striated muscles. It was possible to determine the orientation of the G-actin monomer, which best accounted for the F-actin filament diagram (Holmes *et al.*, 1990) and thus arrive to an atomic model of the filament (Lorenz *et al.*, 1993). Thin filaments also contain a troponin-tropomyosin regulatory complex, which initiates contraction upon influx of Ca^{2+} ions, and nebulin, a long protein, which perhaps regulates the length of actin filaments.

Myosin is a major muscle component comprising about 50% of the total protein of striated muscles. The 520 kDa myosin molecule consists of two heavy chains, two essential light chains (ELC) and two regulatory light chains (RLC). The heavy chains are intertwined in the coiled-coil tail region, which forms the bulk of the thick filament. The tail fragment can be tryptically separated from a globular head region (or subframent-1, S1), which also binds the light chains, one ELC and one RLC per head.

The S1 contains the ATPase site and is responsible for the displacement of actin, operating as a cross-bridge between thick and thin filaments. The heavy chain of S1 can be digested by trypsin into three fragments of 27 kDa, 50 kDa and 20 kDa. These fragments are often used to refer to the respective parts of S1. Myosin polymerizes under physiological conditions. Natural thick filaments consist of several hundred myosin molecules with their tails packed in a staggered array. The thick filaments also contain a long protein titin and a C-protein, which perhaps regulates the structure of the filaments.

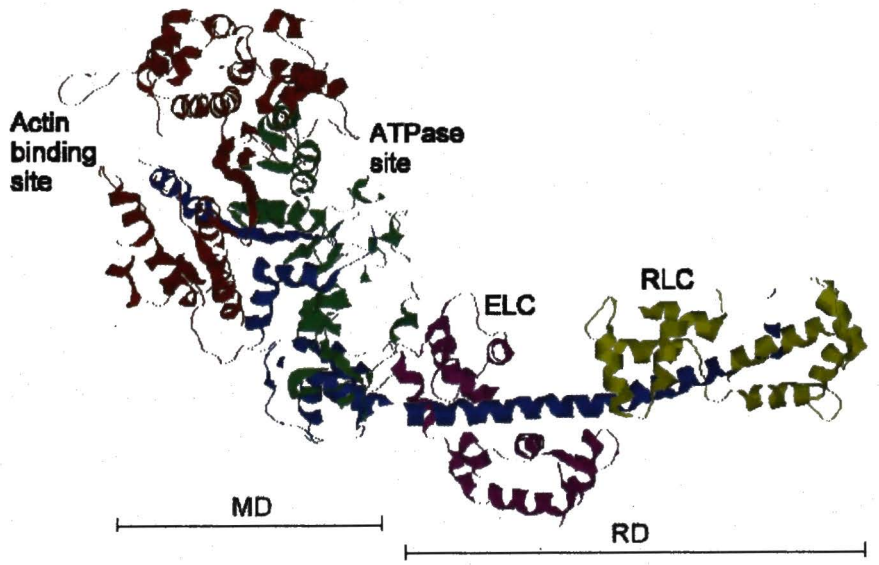

Figure 2: Structure of chicken skeletal muscle myosin subfragment-1 (after Rayment *et al.*, 1993a). The locations of the regions corresponding to the MD and RD are shown. ELC is in magenta; RLC is in yellow. Fragments of the heavy chain, which can be obtained by digestion with trypsin are shown in different colors: 27 kDa – green, 50 kDa – red, 20 kDa – blue.

Rayment *et al.* (1993a) solved the structure of chicken skeletal muscle S1 (Fig. 2). They showed that the 884-residue S1 is "whale"-like

in form and can be roughly divided into motor and regulatory domains (MD and RD, respectively). The MD contains a 7-stranded β-sheet and numerous associated α-helices forming a deep cleft, with the actin binding sites and nucleotide binding sites on opposite sides of the sheet. The cleft separates two parts of the molecule, which are referred to as the 50 kDa upper and 50 kDa lower domains or actin binding domain. The C-terminal RD, which also provides connection to the thick filament, forms an extended α-helix and is wrapped around by a tandem of light chains.

The light chains have a "dumbbell"-like fold, consisting of two lobes joined by a linker. Each lobe contains a pair of helix-turn-helix motifs (EF-hands). Binding to the heavy chain is determined by a special IQ motif in the sequence (Xie *et al.*, 1994). The RLC binds one Ca^{2+} ion and can be phosphorylated by a specific kinase. However, while the phosphorylation triggers contraction in smooth muscles, its role in striated muscles is still obscure. Neither RLC nor ELC affect myosin ATPase activity (Wagner and Giniger, 1981; Sivaramakrishan and Burke, 1982). Moreover, the light chains exist in a dissociation-association equilibrium with the heavy chain (Zaager and Burke, 1988). It was shown that the selective removal of the light chains decreases *in vitro* actin motility (Lowey, 1993a; Lowey, 1993b) and in case of ELC produces 50% smaller force (Van Buren *et al.*, 1994). Therefore it was suggested that the light chains are needed primarily to stabilize the extended conformation of the heavy chain (Lowey and Trybus, 1995).

2. Lever arm model of muscle contraction

Fitting of the atomic structures of F-actin and S1 into three-dimensional cryoelectron microscope reconstructions gave an atomic model of the actomyosin complex (Rayment *et al.*, 1993b), which was later refined (Mendelson and Morris, 1997). In particular, this model establishes the spatial orientation of S1 in the active complex. For example, 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 50 kDa upper and lower domains and the ATP binding site extends from the 50 kDa upper domain into the 50 kDa lower domain. Furthermore, the very extended RD 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" (Houdusse and Cohen, 1996), 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 (Holmes, 1997). Because it is the hydrolysis of ATP that induces the conformational change in myosin, there must be a sensor element, which would recognize the presence and absence of a single phosphate group. The comparison of the S1 structures with and without bound ATP analogs revealed that this element consists of the switch I and switch II loops (Fisher *et al.*, 1995; Smith and Rayment, 1996). According to this model, their movement results in the release of the γ-phosphate of ATP. It has been suggested

that the movement of the switch II loop tilts and translates the relay helix, which links the catalytic and mechanical sites, and thus induces the rotation of the lever arm (Dominguez *et al.*, 1998; Fisher *et al.*, 1995; Smith and Rayment, 1996). Almost identical mechanism exists in kinesins (Vale and Milligan, 2000).

Support for the lever arm hypothesis has been obtained from the electric birefringence experiments (Highsmith and Eden, 1993), which showed that changes in the hydrodynamic radius of S1 upon hydrolysis of ATP could be explained by the changes occurring in the RD. Small angle synchrotron X-ray scattering experiments showed that conformational change occurred in the RD of S1 during the hydrolysis of ATP (Wakabayashi *et al.*, 1992) and upon binding of different ATP analogs (Sugimoto *et al.*, 1995). Similarly, recent studies have suggested that structural (Fisher *et al.*, 1995) and chemical (Phan *et al.*, 1997) differences between the complexes of S1·Mg·ADP carrying different phosphate analogs occur near the C-terminus of the MD. The fluorescent resonance energy transfer studies of two fluorophores attached at different points in myosin on either side of the presumed hinge of the lever arm were consistent with the swinging theory (Suzuki *et al.*, 1998). Also, the light chain region of myosin is capable of motions independent of the catalytic domain (Irving *et al.*, 1995). In view of these findings it is reasonable to expect that part of the muscle power stroke arises from the rotational motion of the RD.

Recently such rotational motion (30-35°) has been observed in high-resolution electron micrographs of actin decorated with smooth

muscle S1 and brush border myosin I upon binding of ADP (Jontes *et al.*, 1995; Whittaker *et al.*, 1995). Crystallographic data (Dominguez *et al.*, 1998) suggest that the orientation of the RD of smooth S1 carrying MgADP·AlF^{3-} (a likely pre-power stroke conformation) differs from the orientation obtained from early Rayment's data of skeletal S1 in rigor (a post-power stroke conformation). However, it has been reported that ADP had no effect on the orientation of S1 from skeletal muscle (Gollub *et al.*, 1996, Borejdo *et al.*, 1997). Time-resolved electron micrograph studies in fact show no bulk change of the cross-bridge orientation on binding ATP before dissociation takes place (Pollard *et al.*, 1993) whereby a reorientation of the lever arm would not have been detected at the attainable resolution.

3. What part of myosin is responsible for the force generation?

There are suggestions that the MD may be involved in the production of force, rather than the RD. Recent results from our laboratory showed that S1 binds to actin in two conformations, differing in the orientation of MD (fig. 3; Andreeva *et al.*, 1993). The mode of binding of skeletal myosin head depends on the molar ratio of S1 to actin (Andreev and Borejdo, 1992). This problem has been extensively studied in recent years by means of carbodiimide (EDC) chemical cross-linking. This cross-linker catalyzes peptide bond formation between ε-amino group of lysine and carboxyl groups of aspartate or glutamate. The method is highly sensitive even to a small change in the relative position of the amino acids (Grabarek and Gergely, 1990).

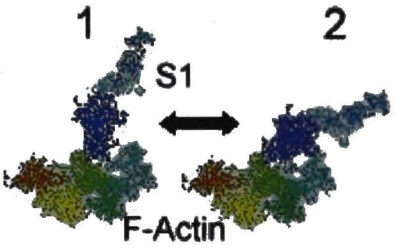

Figure 3: Two-state model of actin-S1 interaction proposed by Andreeva *et al.*, 1993. Five actin monomers are shown in different colors. The MD (shown in blue) of S1 binds to one actin monomer (green) in state 1, and to two actin monomers (green and cyan) in state 2.

Mornet *et al.* (1981) first found that actin and S1 form three different complexes in rigor with the molecular masses of 175, 185 and 265 kDa. The 175 and 185 kDa peptides were shown to contain one actin monomer and one S1 (Sutoh, 1983). The only difference between the two peptides was in the site of cross-linking of S1: Tyr626-Glu647 loop or Lys567-His578, respectively. The 265 kDa complex was suggested to contain two actin monomers and one S1 (Heaphy and Treager, 1984, Mornet *et al.*, 1989). This was later confirmed by Andreev and Borejdo, who also showed that the 1:2 complex has a lower ATPase activity (Andreev and Borejdo, 1992; Andreeva *et al.*, 1993). Moreover, they found that the ratio of 1:1 and 1:2 complexes of S1 to actin strongly depended on the degree of saturation of F-actin with S1. They also studied the kinetics of complex formation (Andreev *et al.*, 1993). The findings led them to a two-state theory of acto-S1 interaction, which proposed that the S1 first binds to one actin monomer (state 1), and then changes its conformation to bind to two actin monomers (state 2) (Andreeva *et al.*, 1993). Such a transformation is possible in skeletal

muscles, where actin is in excess over myosin heads (Morimoto and Harrington, 1974; Squire, 1981).

This theory has an important consequence, since it suggests that it is the conformational change in the MD rather than the rotation of the RD that could be responsible for the production of force. New evidences appeared recently, showing that the MD tilts during the ATP-hydrolysis cycle and may contribute to the power stroke (Berger *et al.*, 1996; Burghardt *et al.*, 1997).

4. The hypothesis: muscle contraction originates from conformational changes in both the regulatory and motor domains of S1

Based on the evidence described above, we hypothesize that the changes in both MD and RD are necessary for the power stroke to occur. To test this hypothesis, we used two approaches. In the first approach, we probed orientation of the MD using EDC cross-linking.

Our results show that MD can be cross-linked to actin at low ratios of S1 to actin. Moreover, ADP does not abolish actin-S1 complex formation, but simply shifts the ratio of S1 to actin at which this occurs. The results are thus consistent with the idea that MD changes orientation during contraction.

In the second approach, we measured orientation of the RD using fluorescence polarization (anisotropy). Placing a fluorescent probe on a protein for the subsequent fluorescence polarization studies is one of the high-resolution methods used to investigate the behavior of actin and

myosin during the cross-bridge cycle. This method has been used in muscle research since late sixties. It provides information on both local and global conformational changes in protein. The Cys707 located in the hinge region of S1 has been typically used as a locus for the thiol-reactive probes. It was found that during muscle contraction the change occurs in spatial organization and mobility of myosin cross-bridges (Nihei *et al.*, 1974; Borejdo and Putnam, 1977). However, no global conformational changes in myosin could be found (Yanagida, 1981, 1984, 1985).

In recent years, the RLC has been widely utilized as a labeling site to explore the possibility of the RD rotation. The RLC is located at the C-terminal end of RD, which is farthest from the MD and therefore the rotation is expected to be large and easily detectable. Moreover, labeling of the RLC does not affect the ATPase activity. It was found that the orientation of RLC changes during rapid length change (Irving, 1995; Hopkins *et al.*, 1998; Sabido-David *et al.*, 1998) and upon sudden generation of ATP [Allen *et al.*, 1996). Moreover, it has been recently shown that the RD does not only tilt, but also twists during muscle contraction (Corrie *et al.*, 1999).

The fluorescence anisotropy (an equivalent of polarization) technique was used to test the rotation of the RD. However, the fluorescent probe was placed at the ELC. This eliminates the negative effect on myosin ATPase, which occurs after labeling Cys707, and facilitates the experiments, since the ELC-containing S1 can be easily obtained and the exchange of ELC into myosin in muscle fibers is more

efficient than the RLC. Moreover, there is virtually no data on the conformation of ELC during the power stroke.

There are two isoforms of the ELC in vertebrate skeletal muscles called LC1 and LC3 according to their relative position on the electrophoresis gel of myosin. The only difference between the isoforms is that the LC1 has 40 more amino acid residues at the N-terminus than the LC3. Apart from the N-termini, the isoforms have identical sequences, since they are produced due to an alternative splicing of the single mRNA transcript (Nabeshima *et al.*, 1984). The crystallographic study of skeletal muscle S1 could not resolve the N-terminus of LC1, because a mixture of isoforms was used and the quality of crystals was poor (Rayment *et al.*, 1993a). However, the difference between the isoforms has a significant consequence for the function. The N-terminal peptide of LC1 is rich in Ala-Pro repeats (Frank and Weeds, 1974), which form an extended structure (Bhandary *et al.*, 1986). This extension enables LC1 to interact with F-actin (Prince et al., 1981; Henry et al, 1985), working as a drag, which makes the muscles slower (Weeds and Taylor, 1975; Lowey *et al.*, 1993; Bottinelli *et al.*, 1994; Sweeney, 1995). It has been recently shown that the positively charged residues at the tip of the N-terminus of LC1 are responsible for the binding to actin (Andreev *et al.*, 1999; Timson *et al.*, 1999). Because the N-terminus of LC1 is relatively independent of the bulk of myosin molecule, it is not clear how it behaves during the cross-bridge cycle. In particular, it might be that the LC1 is still bound to F-actin even when ATP dissociates the heavy chain of myosin from it.

Our results show that both the N- and C-termini of LC1 become immobilized during pre-power stroke and are released during power stroke. These results support the hypothesis that the RD rotates during the power stroke. Overall, our results suggest that both MD and RD are involved in force generation in skeletal muscles.

CHAPTER TWO

METHODS

Both actin and myosin were prepared from the back muscles of rabbit. Adult male rabbits were anesthetized with isofluorane and the muscles were excised and kept on ice prior to grinding in a Moulinex home grinder. All further procedures were done at 4°C.

Preparation of myosin. Myosin was prepared using a procedure described by Tonomura *et al.* (1966). The ground muscles were extracted in 0.3 M KCl, 0.1 M KH_2PO_4, 0.05 M K_2HPO_4, 0.5 mM EDTA, pH 6.5 (3 liters per kilogram of muscle), with a continuous stirring by means of a glass rod. The extract was diluted 12 fold with cold water and filtered through three layers of gauze. The filtrate was diluted with 1.5 volumes of cold water and let to stand for at least 2 hours, until a white precipitate, containing myosin filaments, was formed. The precipitate was centrifuged for 15 minutes at 10,000g. The pellet was resuspended in 0.6 M KCl (final concentration), 1 mM Phosphate buffer, pH 7.5, and stirred for 1 hour on ice to allow complete depolymerization of myosin. The solution was then diluted with an equal volume of water to 0.3 M KCl concentration and centrifuged at 18,000g. The supernatant containing myosin was filtered through 2 layers of wet gauze and centrifuged for 15 minutes at 10,000g. The depolymerization and polymerization procedure was repeated once more, and then the myosin pellet was resuspended in a

minimal volume of 2 M KCl and 1 mM Phosphate buffer, and dialyzed overnight against 0.5 M KCl, 1 mM $NaHCO_3$, pH 7.0. The depolymerized myosin was clarified at 18,000g for 1 hour. The concentration of myosin was measured using an absorption coefficient $A^{1\%}_{280} = 5.6$. The purity was checked by SDS-PAGE. Myosin was used either freshly, within 2 days of preparation, or mixed with 50% glycerol and stored for several months at -20°C.

Actin powder preparation. The residue obtained after filtration of myosin extracted back muscles was used to prepare actin powder according to a procedure by Spudich and Watt (1971). First, the residue was extracted with 0.05 M $NaHCO_3$, pH 7.5 and filtered through four layers of gauze. The residue was then extracted in 1 mM EDTA, pH 6.5 and filtered. Then 5 successive quick extractions in cold acetone, 5 L/kg muscle, were done. After overnight air-drying, the acetone powder was stored at -20°C.

Purification of actin from acetone powder. Acetone powder was extracted at 0°C for 30 minutes in buffer A (2 mM Tris-HCl, 0.2 mM $CaCl_2$, 1 mM DTT, 2 mM ATP, pH 8.0) 20 ml per gram of acetone powder and filtered through a Whatman #1 paper filter and four layers of gauze. The residue was washed again with buffer A (10 ml/g), filtered, mixed with the first filtrate and clarified at 36,000g for 1 hour. 50 mM KCl and 2 mM $MgCl_2$ were added to the supernatant and actin was allowed to polymerize at room temperature for 2 hours. KCl was then added to 0.6 M final concentration and the solution was gently stirred for 1.5 hours at room temperature. Polymerized actin

was pelleted by centrifugation at 100,000g for 2 hours, resuspended by homogenization in buffer A (3 ml/g acetone powder) and dialyzed for 3 days in buffer A to allow depolymerization. G-actin was clarified at 100,000g for 2 hours. The concentration of actin was measured using an absorption coefficient $A^{1\%}_{290}$=6.3 (Yang *et al.*, 1979). The purity was checked by SDS-PAGE. In order to polymerize actin, the concentrations of KCl and $MgCl_2$ were brought to 50 mM and 2 mM, respectively. F-actin was stored at 0°C and used within a month.

Myosin Subfragment-1 preparation. Myosin S1 was prepared by chymotryptic digestion of myosin according to Weeds and Pope (1977) and Weeds and Taylor (1975), with minor modifications.

Myosin was dialyzed overnight against 10 mM phosphate buffer, pH 7.0, 0.2 mM DTT, 1 mM $CaCl_2$, 0.125 mM NaCl. Digestion was conducted at 25°C for 10 minutes with 1:500 w/w α-chymotrypsin to myosin. The reaction was stopped by addition of 1 mM PMSF. The protein was dialysed against 40 mM NaCl, 5 mM phosphate buffer, pH 6.5 at 4°C to polymerize cleaved myosin tail and undigested myosin. Polymerized protein was removed by centrifugation at 40,000g for 90 minutes and the concentration of S1 in the supernatant was measured using extinction coefficient $A^{1\%}_{280}$=7.5 (Ando, 1987). The quality of preparation was checked by SDS-PAGE.

Separation of S1(LC1) and S1(LC3) isoforms was carried out on DE52 ion exchange column according to Weeds and Taylor (1975).

Preparation of myosin essential light chains 1 and 3. The LC1 and LC3 isoforms of essential light chain were prepared using rabbit

skeletal muscle myosin according to Holt and Lowey (1975). The concentration of LC1 was measured as $A^{1\%}_{280}$=2.3 (Marsh and Lowey, 1980), and LC3 as $A^{1\%}_{280}$=1.9 (Holt and Lowey, 1975).

Expression and isolation of the regulatory light chain (RLC). In order to express and purify RLC, a procedure described by Wolff-Long *et al.* (1993) was used. A one liter culture of BL21(DE3)pLysS *E.coli* (B, F-, dcm, ompT, hsds(r_B-m_B-), *gal* λ(DE3), [pLysS Cam^r]) containing pT7-7-LC2Cys73 construct for the chicken fast skeletal muscle RLC (MLC2 gene) with a single Cys73 (Saraswat and Lowey, 1991), provided by S. Lowey (Univ. Vermont), were grown for 14-16 hours at 37°C in enriched buffered media (2% bacto-tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol, 50 mM potassium phosphate, pH 7.2, 100 μg/ml ampicillin, 20 μg/ml chloramphenicol). Cells were pelleted at 8,000g for 10 minutes and washed once with 25 mM Tris-HCl, pH 8.0, 5 mM EDTA. After resuspension in lysis buffer (25 mM Tris-HCl, pH 8.0, 5 mM EDTA, 50 mM glucose, 0.2 mg/ml lysozyme) and incubation on ice for 1 hour, the cells were frozen in liquid nitrogen and allowed to thaw to 4°C. Magnesium chloride was added to 10 mM for DNase I treatment (5 μg/ml, 60 min on ice). Triton X-100 was added to 0.1%, and the inclusion bodies, which contain RLC were pelleted at 15,000g for 15 min. The pellet was washed three times in lysis buffer/Triton excluding lysozyme, after which a final wash was performed in the absence of detergent. The pellet was solubilized in 6 M guanidine-HCl in PBS (20 mM phosphate buffer, pH 7.0, 150 mM NaCl, 3 mM sodium azide) containing 10 mM DTT and stirred gently for 1 hour at room temperature. Insoluble debris was removed by

centrifugation at 100,000g for 30 minutes, and the supernatant was dialyzed against PBS with 1 mM DTT at 4°C with several changes of buffer. Irreversibly denatured proteins were removed by centrifugation at 100,000g for 30 minutes, and the protein in the supernatant was precipitated with ammonium sulfate (70 g/100 ml) and collected by centrifugation at 60,000g for 20 minutes. The final pellet was resuspended in PBS with 1 mM DTT and dialyzed into 10 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM sodium azide at 4°C for further purification by hydroxylapatite (Bio-Rad) chromatography. The sample was applied to a hydroxylapatite column (1.5x10 cm) equilibrated in the same buffer and eluted with a linear gradient of sodium phosphate from 10 to 250 mM (total volume 300 ml). Fractions containing RLC were identified by SDS-PAGE and dialyzed against 5 mM sodium phosphate, pH 7.0, before lyophilization in the presence of an equal weight of sucrose as cryoprotectant. Freeze-dried RLC powder was stored at –20°C.

Expression and isolation of essential light chain 1 containing single cys2 (LC1m). The C178A cDNA was cloned by L. Saraswat and S. Lowey (Univ. Vermont) into the *Eco*RI site of pT7-7 vector to produce the chicken fast skeletal muscle LC1 (MLC1 gene) with an N-terminal tag containing cysteine (ACGI) followed by the native LC1 sequence starting at Pro2 instead of Ala. The sequence was ACGI-PKKDVKKPAAAAAPAPAPAPAPAPAPAKPKEPAIDLKSIKIEFSKEQQDD...

The BL21(DE3) cells containing pT7-7-LC1Cys2 construct were grown and lysed as described for the RLC. In contrast to RLC, the ELC does not form inclusion bodies. Therefore, 6 M guanidine-HCl

was added directly to the lysate. All further purification was identical to the RLC. After the hydroxylapatite column, ELC containing fractions were pooled and dialyzed against 10 mM imidazole, pH 7.0, 20 mM NaCl, 1 mM EDTA, 1 mM DTT and 3 mM NaN_3. The protein was then applied on a 1x15 cm DE52 column and eluted with a linear gradient of 20-250 mM NaCl. Fractions containing ELC were identified by SDS-PAGE and dialyzed against 5 mM sodium phosphate, pH 7.0, before lyophilization in the presence of an equal weight of sucrose as cryoprotectant. Freeze-dried RLC powder was stored at –20°C.

His-tag essential light chain 1 (LC1) cloning, expression and purification. The pUC8-hA5-13 cDNA clone for the human fast skeletal muscle myosin light chain 1 (MYL1 gene, Seidel *et al.*, 1988) was purchased from ATCC (Cat. №59696). To facilitate purification of the protein, a tag of 6 histidine residues was introduced at the C-terminus of LC1 by polymerase chain reaction. The LCPC1 and LCPC3 primers, synthesized by IDT DNA, were used, with the downstream primer containing a His-tag sequence just before the stop codon:

Up (LCPC1) – 5'-taa aaa acc *atg* gca cca aag aaa gac

Down (LCPC3) – 5'-gtt tgc aga tct *tta* gtg atg gtg atg gtg atg gat aga cat cat gtg ctt gac

The further steps were carried out as widely described (Ausubel *et al.*, 1995; Sambrook *et al.*, 1989). After 45 cycles of Taq-polymerase amplification (1 minute at 94°C, 2 minutes at 55°C and 7 minutes at 72°C) the LC1 PCR product was isolated by low melting point agarose

gel electrophoresis and then treated with *Nco*I and *Bgl*II for 4 hours at 37°C.

The pQE-60 vector (QIAGEN) was used for cloning. The vector was treated with *Nco*I and *Bgl*II and then purified by low melting point agarose gel electrophoresis. The vector DNA and LC1 insert were ligated with T4 DNA ligase. M15[pREP4] *E.coli* (Nal^S, Str^S, Rif^S, Lac^-, Ara^-, Gal^-, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+, [pREP4]) $CaCl_2$ competent cells were transformed with the ligation mixture and then plated on LB agar, containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. After growing overnight at 37°C single cell colonies were transferred into LB media containing antibiotics and later used for plasmid DNA analysis and expression of MYL1. To verify the changes made in MYL1, pQE-60-LC1 was isolated using Plasmid DNA Minipreparation kit (QIAGEN) and sequenced at the DNA Sequencing Facility (Iowa State Univ.).

To express LC1, 20 ml of overnight cell culture were transferred to 1L of LB media containing 100 μg/ml ampicillin and 25 μg/ml kanamycin and grown to OD_{450} = 0.5. Then the expression was induced by addition of 1 mM IPTG. After 4 hours of expression the cells were collected by centrifugation at 4,000g for 20 minutes, frozen in liquid nitrogen and stored at –20°C until purification.

Batch purification under denaturing conditions recommended by QIAGEN was used to isolate expressed LC1. The frozen cell pellet was incubated on ice for 15 minutes and then resuspended in buffer A (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0) at 5 ml per gram wet weight. The remainder of the purification was conducted at room temperature. The cells were stirred for 60 minutes and then centrifuged at 10,000g for 20 minutes. One ml of 50% Ni-NTA agarose

slurry (QIAGEN) was added to 4 ml of cleared lysate and mixed by gentle shaking for 60 minutes. The lysate-resin mixture was loaded into empty column (1.5x10 cm) and the flow-through was collected. The column was washed with 2 volumes of buffer B (same as buffer A, but pH 6.3). The LC1 was eluted with 1 volume of buffer C (same as buffer A, but pH 5.9) and then with 1 volume of buffer D (same as buffer A, but pH 4.5). The purified LC1 was analyzed by SDS-PAGE and used for labeling or freeze-dried in the presence of an equal weight of sucrose and stored at –20°C.

Preparation of muscle fibers and myofibrils. Isolated muscle fibers were prepared from glycerinated rabbit *psoas* muscle bundles by dissecting single fibers in glycerinating solution and attaching the ends of the tautly stretched fiber to the aluminum clips mounted on the microscope slide. Mounted fibers were thoroughly washed with rigor solution and covered with a cover slip. To make myofibrils, the bundles of fibers were transferred from glycerinating solution to a large volume of EDTA-rigor solution for 1/2 hr, and then to rigor solution. Fibers were homogenized in cold rigor solution using 8 setting of the Omni Mixer (Waterbury, MA) for 30 sec. After homogenization, the myofibrils were filtered through 2 layers of gauze, and 0.1 mM PMSF was added to prevent proteolysis.

Tricine Sodium-Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis and Western blots. Gel electrophoresis was carried out according to Schagger and von Jagow (1987) using 8% polyacrylamide gels. After electrophoresis the peptides were electroblotted onto

nitrocellulose membranes (Bio-Rad). Membranes were incubated for 1 hour with a blocking solution, then for 1 hour with the primary monoclonal antibodies, and finally for 1 hour with the horseradish peroxidase-conjugated secondary antibodies (ECL, Amersham). Luminescence was detected by X-ray film. Film was scanned in a transmission mode (transmission arm #01719) on a SM3+ Howtek scanner (Hudson). The Image Pro Plus program (Media Cybernetics) was used to measure the relative intensity of the various bands.

Labeling of light chains. The isolated ELC was labeled by incubation with 5 molar excess of 5'-IATR for 4 hrs in 50 mM KCl, 2 mM EDTA, 10 mM Phosphate buffer pH 7.0 at 4^{0}C. The RLC was labeled with 5'-IATR or 5'-IAF at room temperature for 24 hrs. The free dye was removed by passing through a Sephadex-50 column, and samples were dialyzed against rigor solution.

Exchange of light chains into S1. Fluorescently labeled LC1, LC1m or LC3 were exchanged into S1(LC3) under mild denaturing conditions as described by Wagner and Weeds (1977). S1 was purified on a DE-52 column to separate the unexchanged S1(LC3). The concentration of labeled S1 was calculated from the extinction coefficient of rhodamine at 280 ($\varepsilon^{1\%}$=18,750) and 555 nm ($\varepsilon^{1\%}$=75,000) as $C_{S1}(mg/mL)=(A_{280}-A_{555}/4)/0.75$ and the concentration of the dye as $C_{IATR}(mM)=A_{555}x10^3/75.0$. Typically, 30-40% of S1 was labeled.

Exchange of light chains into fibers. The labeled LC1 or LC1m were exchanged with endogenous light chains of myosin in muscle

fibers at 30°C using the exchange solution described by Sweeney (1995). The concentration of TFP was decreased to 100 μM to reduce direct effects of TFP on ELC (Huang *et al.*, 1998) and on RLC (Malmqvist *et al.*, 1997). No EDTA was used. After washing with EDTA-rigor, fibers did not contract in relaxing solution, suggesting that the exchange procedure resulted in only limited extraction of the regulatory proteins. For this reason, the fibers were not irrigated with troponin, troponin C or RLC. The degree of labeling was estimated by comparing the intensity of fluorescence of fibers that underwent exchange, with the intensity of known concentration of labeled light chains. Concentration of myosin in the fibers was taken as 120 μM (Bagshaw, 1982). Typically, 5-8% of cross-bridges were labeled. The RLC was exchanged into fibers as previously described (Ling *et al.*, 1996).

EDC cross-linking. S1 and F-actin were mixed at different molar ratios and incubated for ½-1 hr at room temperature; appropriate amounts of 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) were then added. The reactions were stopped by adding an equal volume of electrophoresis sample solution (4% SDS, 24% glycerol, 100 mM Tris, 4% mercaptoethanol, 0.02% Bromphenol Blue). Unless otherwise indicated all cross-linking experiments were done in solutions containing 0.2 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 7.5. The low concentrations of $MgCl_2$ and KCl were used to prevent the formation of actin filament bundle (Andreev and Borejdo, 1992; 1995).

In case of the two-step cross-linking (Grabarek and Gergely, 1990) actin was preactivated with 20 mM N-hydroxysuccinate (NHS) and 10 mM EDC. The reaction was stopped with 0.1 M β-mercaptoethanol after 20 min. Aliquots of preactivated actin were added to S1. The cross-linking reaction was stopped by addition of an equal volume of electrophoresis sample solution.

Fluorescence anisotropy of free S1 and light chains. Polarization (anisotropy) is a result of the photoselection of the fluorophores by their orientation with respect to the direction of the polarized excitation. The rotational diffusion of fluorophores can depolarize the emitting light. Measurement of polarization elicits the angle shift of the fluorophore, which occurs between the adsorption and emission of a photon. The angle shift depends on the speed and degree of the rotational diffusion during the lifetime of the excited state. The rotational diffusion depends on the viscosity of the solvent as well as on the size and shape of the diffusing particle (Lakowicz, 1986).

Steady-state anisotropy (r) was measured using an SLM 500C spectrofluorometer (Spectronic Instruments). 5'-IATR was excited at 530 nm and r was measured at 580 nm, with excitation and emission slits at 5 and 10 nm, respectively. Excited state lifetimes (τ) and rotational correlation times (ρ) were measured on an ISS-K2 digital multifrequency fluorometer (ISS Inc) using 510 nm excitation.

Fluorescence anisotropy measurements in muscle fibers. The anisotropy of rigor fibers was measured as described earlier (Xiao *et al.*, 1995) using argon ion laser operating at 514.5 nm (Spectra Physics Model 164) or a green He-Ne laser operating at 543 nm

(Melles-Griot, Model 05-LGP-193) as an excitation source. Before applying relaxing solution, fibers were washed with EDTA-rigor. Otherwise, fibers contracted due to the insensitivity of the regulatory system at low ATP concentrations (Weber and Murray, 1973). The dry long distance objective Zeiss UD 40x was used. A birefringent crystal split the emitted light into ordinary and extraordinary rays. The anisotropies are:

$$r_{\parallel}=({}_{\parallel}I_{\parallel}/C_{\parallel}-{}_{\parallel}I_{\perp}\}/\{{}_{\parallel}I_{\parallel}/C_{\parallel}+2{}_{\parallel}I_{\perp}\}$$
$$r_{\perp}=\{{}_{\perp}I_{\perp}/C_{\perp}-{}_{\perp}I_{\parallel}\}/\{{}_{\perp}I_{\perp}/C_{\perp}+2{}_{\perp}I_{\parallel}\}$$

where left and right subscripts indicate the direction of the polarization of the exciting and emitted light with respect to fiber axis, and the variables 'c' are correction factors. The correction factors to eliminate the systematic error due to a more efficient transmission by the dichroic mirror of the horizontally polarized light were defined as $C_{\parallel}={}_{\parallel}I_{\parallel}/{}_{\parallel}I_{\perp}=0.70$ and $C_{\perp}={}_{\perp}I_{\perp}/{}_{\perp}I_{\parallel}=1.54$.

Confocal microscopy. Confocal Laser Scanning Microscope LSM 410 (Carl Zeiss) mounted with water immersion Zeiss Planochromat Korr W 40× objective was used to study the localization of light chains exchanged into muscle fibers. 5'-IATR and 5'-IAF were excited with argon laser at 568 nm and 488 nm, and emission was registered at 590 nm and 515 nm, respectively.

CHAPTER THREE

RESULTS

1. ADP causes reorientation of S1 motor domain on F-actin

Recently, it has been shown in our laboratory that the binding of S1 to F-actin depends on the molar ratio of S1 to actin. It was found that at high ratio of S1 to actin the formation of actin:S1:actin complexes was inhibited. We extend those findings to show that the binding of S1 to one or to two actin monomers depends not only on the molar ratio, but also on the phosphorylation states of adenine nucleotides.

a. The effect of ADP on cross-linking

In order to investigate the effect of ADP, AMP-PNP and pyrophosphate (PPi) on the formation of complexes, skeletal muscle S1 and actin were mixed at different ratios. The results of cross-linking in the presence of 2 mM ADP are shown in Figure 4A. At low ratio S1:actin the following major bands are visible (in order of decreasing molecular masses): 235, 210, 185, 150, 160, 120, 95, 66, 43 and 24 kDa. In agreement with earlier work the formation of the 235, 210, 185, 160 and 66 kDa complexes was inhibited at high S1:actin molar ratios (Andreev and Borejdo, 1995). The comparison of Coomassie blue staining (Fig. 4A), Western blots using antibodies against LC1 (Fig. 4B), the fluorescence patterns obtained with fluorescent actin

(Fig. 4C) and fluorescent S1 (not shown) suggests that the bands correspond to the following complexes - 235 kDa: HC+A+A+LC1, 210 kDa: HC+A+A, 185 kDa: HC+A+LC1, 160 kDa: HC+A (cross-linked through a site on a 50 kDa tryptic fragment), 150 kDa: HC+A (cross-linked through a site on a 20 kDa tryptic fragment), 120 kDa: HC+LC1, 66 kDa: A+LC1, 95 kDa: HC, 43 kDa: A, 24 kDa: LC1 (Fig. 5).

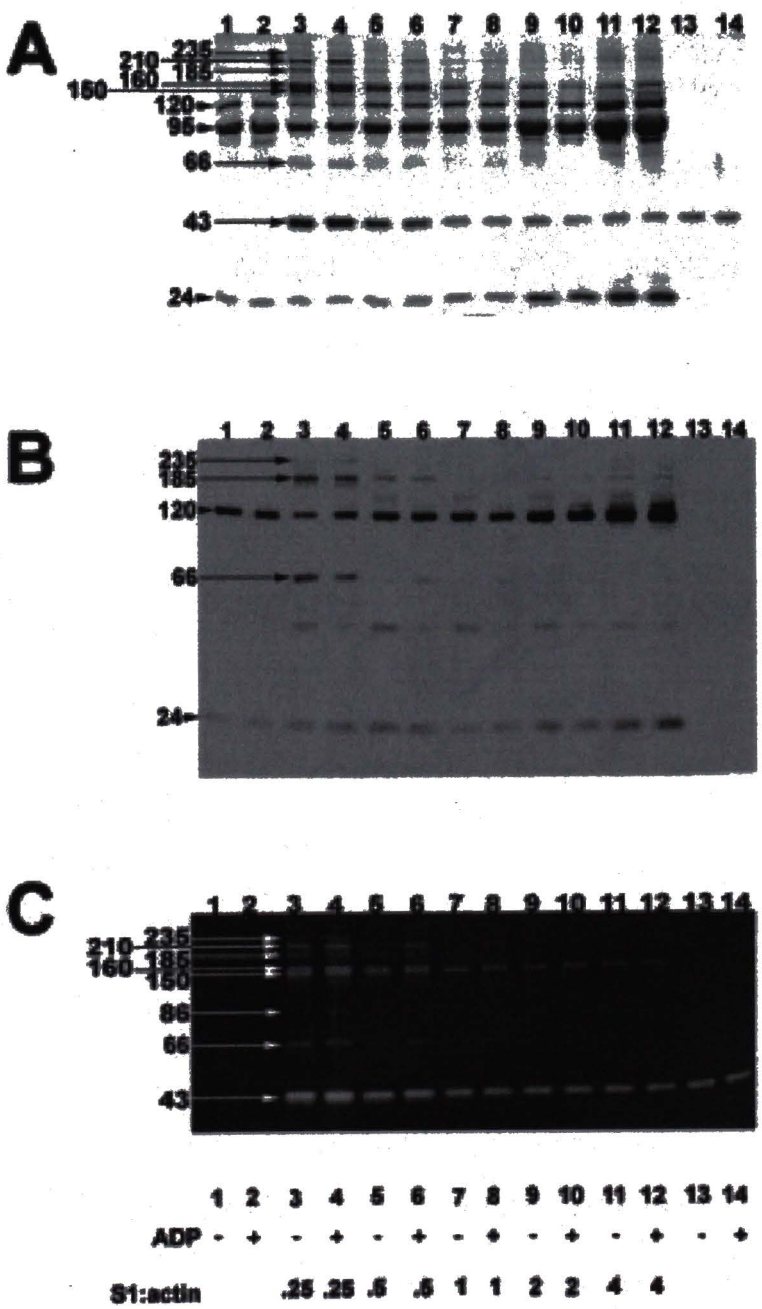

Figure 4: Effect of ADP on cross-linking of skeletal S1 with F-actin at different molar ratios. Cross-linking was performed with 75 mM EDC for 40 min at room temperature in 50 mM KCl, 0.5 mM $MgCl_2$, 10 mM Tris-HCl buffer pH 7.5, 23°C. A: 8% Polyacrylamide SDS Tricine gel stained with Coomassie blue. B: Western blot of the same gel using antibodies against LC1, C: Fluoresence picture of the same gel, actin fluorescently labeled at Gln-41 with dansyl cadaverine-rhodamine according to Takashi (1988). All lanes contain EDC. Lane 1: S1(LC1), lane 2: same as in lane 1 + 2mM ADP, lane 3: 8 μM actin+2μM S1, lane 4: same as lane 3 +2 mM ADP, lane 5: 4 μM actin+2 μM S1, lane 6: same as lane 5 + 2 mM ADP, lane 7: 4 μM actin+4 μM S1, lane 8: same as lane 7 + 2 mM ADP, lane 9: 4 μM actin+8 μM S1, lane 10: same as lane 9 + 2 mM ADP, lane 11: 2 μM actin+8 μM S1, lane lane 12: same as lane 11 + 2 mM ADP, lane 13: 2 μM actin, lane 14: same as lane 13 + 2 mM ADP. ADP concentration was 2 mM ADP and it contained 1 mg/mL hexokinase+100 mM glucose.

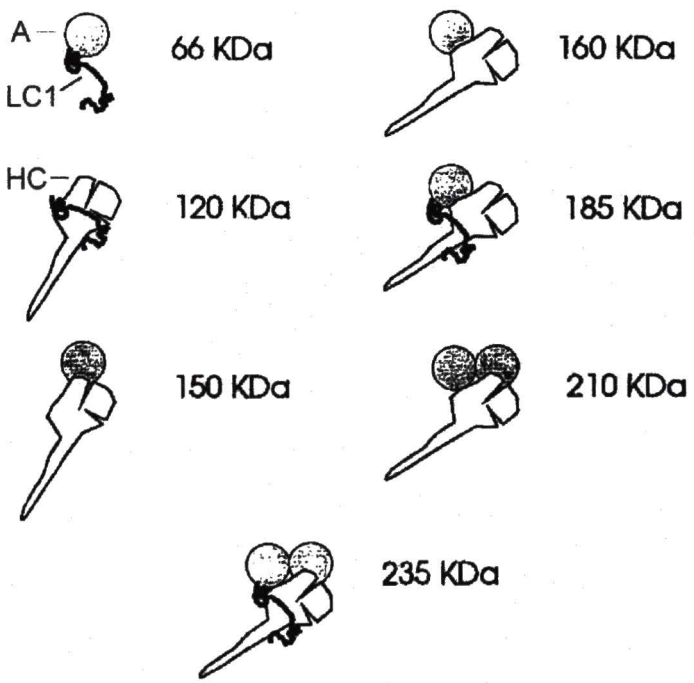

Figure 5: Complexes of S1 and F-Actin formed by EDC cross-linking. A- Actin; LC1 – light chain 1; HC – heavy chain.

The effect of ADP on the formation of adducts at different molar ratios of S1(LC1):actin was examined in 3 experiments using different protein preparations. Qualitatively, ADP had the same effect in each experiment. Quantitatively, the least accurate measurement was that

of the intensity of the 210 kDa band at the molar ratio of 1. Even in this case, however, the ratio R of the intensity in rigor to the intensity in the presence of ADP could be determined with 6% accuracy (the average value of R was 0.73±0.04). The 95% confidence interval was 0.17. The same experiment was carried out with S1(LC3). In this case, the bands corresponding to HC+A+A+LC1 (235 kDa), HC+A+LC1 (185 kDa) and A+LC1 (66 kDa) were absent. The 95% confidence interval was 0.19. Therefore all R values smaller than 0.83 or larger than 1.17 (for S1(LC1), and smaller than 0.81 or larger than 1.19 (for S1(LC3)), were considered significant. The data is summarized in Table 1. Deviations from R=1 that are statistically significant are italic in bold.

Table 1. Ratio of the intensities of the various bands in the S1-actin cross-linking experiment in the presence and absence of ADP.

		R (rigor:ADP ratio)				
band	adduct	0.25 MR	0.5 MR	1 MR	2 MR	4 MR
Data from cross-linking of S1(LC1) with actin						
235	HC+A+A+LC1	0.96	NM[a]	NM	NM	NM
210	HC+A+A	0.88	***0.70***[b]	***0.73***	***0.80***	1.02
185	HC+A+LC1	1.12	1.00	NM	NM	NM
160	HC+A	1.02	***0.82***	NM	NM	NM
150	HC+A	1.06	1.09	0.97	1.12	0.96
120	HC+LC1	0.95	1.01	1.00	0.90	1.02
95	HC	1.00	1.01	1.04	***0.78***	0.99
66	A+LC1	0.97	0.84	***0.71***	NM	NM
43	A	1.00	1.06	0.99	1.00	1.00
24	LC1	0.96	0.99	1.05	0.83	1.02
Data from cross-linking S1(LC3) with actin						
210	HC+A+A	1.02	***0.81***	***0.77***	0.83	1.12

[a] NM indicates that the intensity was too weak to be measured. This is because the formation of the 235, 185, 160 & 66 kDa bands is inhibited at high S1:actin (see text). [b] Statistically significant deviations from 1 are bold and italic.

Table 1 shows that ADP had no effect on the intensity of 1:1 complex (150 kDa) at all ratios of S1 to actin, and the adducts indicative of 1:2 complex (210, 160 and 66 kDa) at a molar ratio of

0.25 (Fig. 1, lanes 3 and 4) and 4 (lanes 11 and 12). However, ADP significantly increased the formation of 210 and 160 kDa adducts when the cross-linking was carried out at a ratio of 0.5 (lanes 5 and 6). Similarly, ADP significantly increased the formation of 210 and 66 kDa adducts when the cross-linking was carried out at a ratio of 1 (lanes 7 and 8) and 2 (lanes 9 and 10). The significance of these results will be considered in the Discussion.

b. The effect of AMP-PNP and PP_i on cross-linking

As a control the effects of AMP-PNP and PP_i agents that have stronger dissociating action than ADP, were examined (Highsmith, 1976; Trybus and Taylor, 1982; Duong *et al.*, 1987a). The pattern of cross-linking is shown in Figure 4.

The assignment of bands is based on the assumption that the bands are equivalent to those seen in the ADP experiments. The intensity of an adduct indicative of 1:1 binding (150 kDa) decreased at all ratios. The intensities of adducts indicative of 1:2 binding (210, 160 and 66 kDa) were more complex. At a high ratio, the intensity of the 210 kDa adduct was 14% greater in the presence of AMP-PNP (Fig. 6A, lane 10) than in its absence (Fig. 6A, lane 9), and its intensity was 11% greater in the presence of PP_i (Fig. 6B, lane 7) than in its absence (Fig. 6B, lane 8).

Conversely, at a low molar ratio the intensity of the 210 kDa adduct was 55% less in the presence of AMP-PNP (Fig. 6A, lane 2) than in its absence (Fig. 6A, lane 1), and its intensity was 31% less in the presence of PP_i (Fig. 6B, lane 1) than in its absence (Fig. 6B, lane 2). At intermediate ratios the intensities of 210 and 160 kDa adducts

decreased. The significance of these findings will be dealt with in the Discussion.

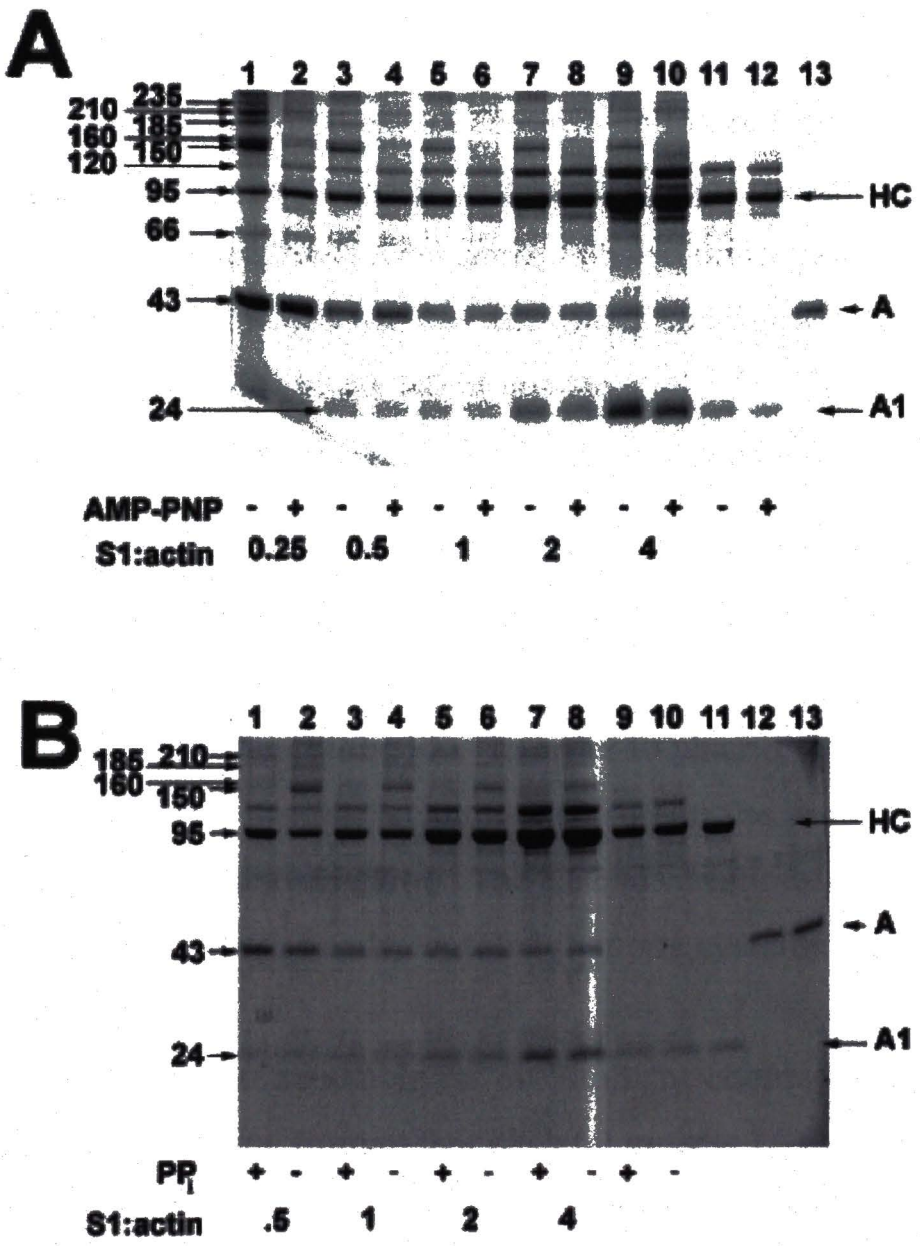

Figure 6: The effect of AMP-PNP (A) and PP_i (B) on cross-linking of F-actin with skeletal S1 at different molar ratios. Conditions as in Fig. 4. The assignment of bands is based on the assumption that the bands are equivalent to those seen in the ADP experiments. The molecular mass, in kDa, of the cross-linked adducts are at left. AMP-PNP and PP_i was either absent (-) or present (+). The molar ratio S1:actin are indicated at the bottom. Abbreviations: HC - heavy chain of S1, A - actin; A1 - essential light chain 1.

2. ATP hydrolysis changes conformation of the regulatory domain

The lever arm hypothesis of actomyosin interaction suggests that the RD, rather than the MD, is responsible for the force production. We used fluorescent probes placed on myosin light chains in order to find out whether the power stroke involves conformational change in the RD of myosin.

a. Preparation of recombinant myosin light chains

Isolated light chains can be readily exchanged with the light chains of S1 or myosin. Moreover, they can be easily expressed in high levels in *E.coli*. This makes the light chains a perfect tool for the studies of conformation of the RD. In order to place a thiol-reactive fluorescent probe in specific location of a protein, the protein must contain a single cysteine residue. The ELC of vertebrate animals has an advantage, since it already contains just one cysteine located near the C-terminus.

Three species of recombinant myosin light chains were used in exchange experiments: human fast skeletal muscle LC1; chicken fast skeletal muscle LC1 (LC1m), with a single cysteine residue moved from position 177 to position 2; and chicken fast skeletal muscle RLC, with a genetically introduced single Cys73.

To facilitate purification, a tag of 6 histidines was introduced at the C-terminus of LC1 by polymerase chain reaction. The pQE-60 vector was used for cloning. This vector has an optimized phage T5

promoter and two *lac* operator sequences, which increase *lac* repressor binding and ensure efficient repression of the powerful T5 promoter (Fig.7). Combination of pQE-60 vector with M15[pREP4] *E.coli* strain permits a high level expression of the recombinant protein.

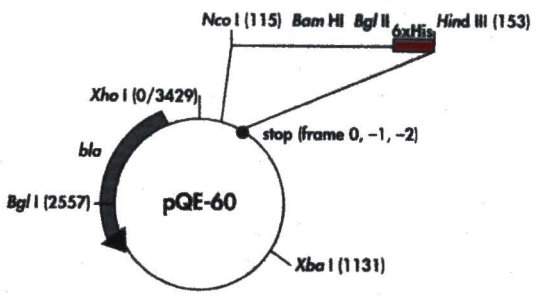

Figure 7: pQE-60 vector. The LC1-his tag DNA was inserted using *Nco*I and *Bgl*II restriction sites of the polylinker.

Restriction analysis of the pQE60-LC1 construct confirmed the presence of the 600 bp LC1 insert (Fig. 8). The DNA sequencing showed that there were no unwanted mutations in LC1. The time-course of LC1 expression is shown on Figure 9. The expression was at the maximal level 3-4 hours after IPTG induction. The LC1 was expressed in a high level (about 50 mg/L).

The his-tag LC1 was readily purified using Ni-NTA agarose under denaturing conditions. Figure 10 shows the steps and quality of purification. An aggregate of LC1 formed when the protein was in high concentration. However, it could be removed by reduction with β-mercaptoethanol. The LC1 was later labeled with either 5'-IATR or 5'-IAF.

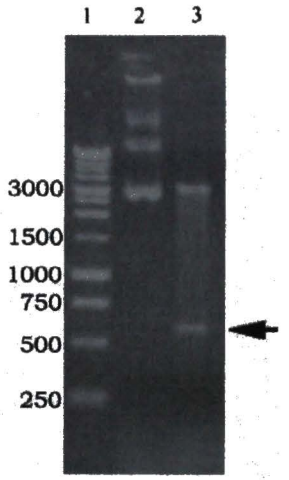

Figure 8: Restriction analysis of pQE-60-LC1 construct. Plasmid DNA minipreps were digested with *NcoI* and *BglII* and the presence of the 600 bp insert containing his-tag LC1 (indicated by arrow) was checked on 1% agarose gel. Lane 1 - molecular weight markers; lane 2 – unrestricted miniprep DNA; lane 3 – the construct DNA after restriction.

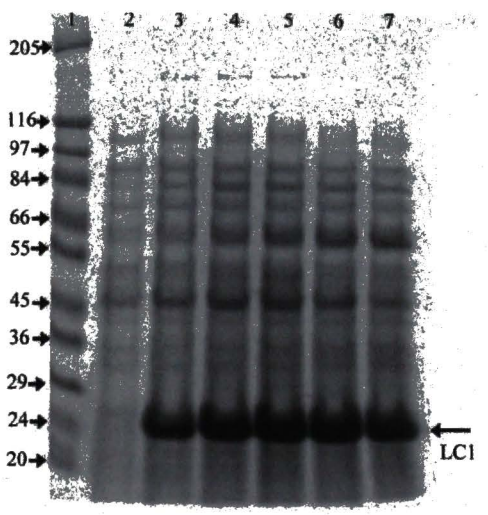

Figure 9: Time-course of LC1 expression. A clone of M15[pREP4][pQE-60-LC1) was grown to OD_{450} = 0.5. Expression was initiated by 1 mM IPTG and 1 ml samples were taken during expression for analysis on 8% SDS-PAGE. Identical volumes of each sample were loaded on the gel. Lane 1 – molecular weight markers, 2 – cell lysate before induction, 3-7 – cell lysates 1, 2, 3, 4, and 5 hours after induction. The position of LC1 is indicated by arrow.

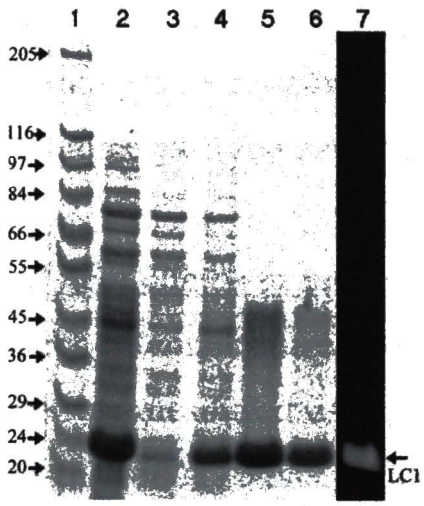

Figure 10: Isolation and labeling of LC1. Lane 1 – molecular weight markers. The cleared cell lysate was loaded on Ni-NTA column at pH 8.0 (lane 2). The free volume did not contain LC1 (lane 3). The column was washed with pH 6.3 buffer (lane 4) and the LC1 was eluted in two steps with pH 5.9 and 4.5 buffers (lanes 5 and 6, respectively). The LC1 was then labeled with 5'-IATR or 5'-IAF (lane 7).

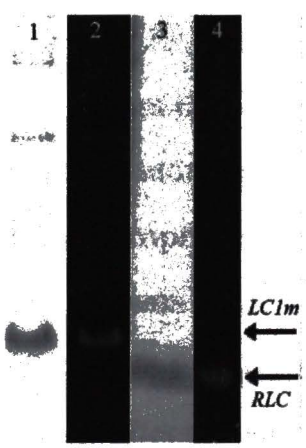

Figure 11: Quality of purified and 5'-IATR labeled LC1m and RLC. Lanes 1 and 3 – 8% SDS-PAGE, and lanes 2 and 4 – fluorescent images of LC1m and RLC, respectively.

Both LCm and RLC cDNA's were previously prepared and provided by S. Lowey, Univ. Vermont. In both cases the light chain coding sequence was inserted into the *Eco*RI restriction site of the

pT7-7 vector and the expression was done in BL21(DE3)pLysS cells. Figure 11 shows the quality of purified and 5'-IATR labeled LC1m and RLC. The LC1m contained minor contaminations. However, they were removed during exchange with the heavy chain.

b. Exchange of light chains in muscle fibers.

To estimate the effect of temperature and TFP on fibers, maximum isometric force was measured using sensitive tension transducer (Rapp and Guth, 1988). Small bundles of fibers were mounted between the force transducer and the rigid arm, and force was measured as previously described (Tang *et al.*, 1992). In 3 experiments, the incubation in relaxing solution at 37°C for 20 minutes (without TFP) caused the tension to decrease on the average by 55.5 ± 4.9%. Subsequent incubation with exchanging solution containing 100 μM TFP at 37°C for 20 minutes caused no further decrease in tension (average decrease 2.5 ± 2.5%). Thus it seems that 100 μM TFP does not have any influence on tension, but that the initial decrease is due solely to raising temperature to 37°C.

Since the degree of labeling was small, the tension alone is not a sensitive measure of muscle function. We suggested that temperature and TFP might affect the orientation of the RD without affecting the tension. To test this, the orientation of the RLC incorporated into muscle fiber was compared with and without TFP. When the exchange of RLC was performed at 30°C, $r_{\perp}$ was 0.235±0.023 and $r_{\parallel}$ was 0.202±0.017. At 37°C these values increased to 0.316±0.013 and 0.230±0.011,

respectively. Exchange at 37°C in the presence of 0.1 mM TFP gave statistically the same values as the exchange at 37°C alone ($r\perp$=0.310±0.008, $r\parallel$=0.221±0.024). It can be concluded that the incubation at 37°C, not TFP, is responsible for the change in polarization.

Exchange at lower temperature is essential for the functional integrity of the fibers. Earlier experiments indicated that the exchange at lower temperature was feasible (Mathew *et al.*, 1998). In our hands, 100 μM TFP at 30°C was 2.5 times less efficient, than 100 μM TFP at 37°C. 1 mM TFP at 30°C was twice as efficient as 100 μM TFP at 37°C. As mentioned above, the fibers exchanged at 30°C with 100 μM TFP were used.

c. Specificity of myosin light chain exchange in muscle fibers.

The specificity of light chain hybridization with myosin heavy chain in muscle fibers was checked by fluorescence intensity and confocal microscopy. The LC1 was exchanged by incubation at 37°C for 30 minutes in relaxing solution in the presence of 100 μM TFP.

Table 2. Non-specific binding of LC1 and RLC in skeletal muscle fibers.

Sample	Intensity, arbitrary units
LC1 exchange	39281±4641
LC1 control	4250±955
RLC exchange	1526±284
RLC control	496±122

The RLC was exchanged by incubation at 30°C for 30 minutes in rigor solution. To estimate non-specific binding, the LC1 or RLC were incubated with muscle fibers at room temperature for 30 min in rigor solution. The degree of exchange was about 10% and 30% of

that in normal exchange conditions (Table 2), for LC1 and RLC, respectively.

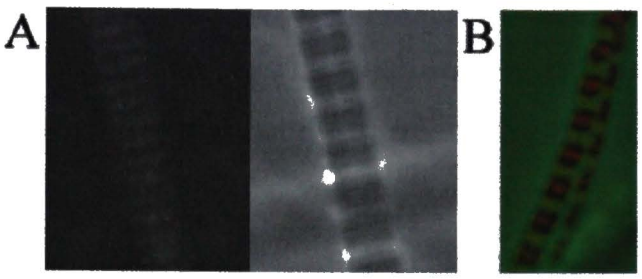

Figure 12: Localization of LC1 in myofibrils. The LC1 was exchanged in relaxed muscle fibers for 30 min at 37°C in the presence of 100 μM TFP. Then the fibers were transferred to rigor and the myofibrils were prepared as described in Methods. A: pattern of rhodamine fluorescence (left) and the view of the same myofibril in transmitted light (right). B: Overlaid images of a myofibril in transmitted light (green) and its rhodamine fluorescence (red). LC1 bound predominantly in the middle of the A-band.

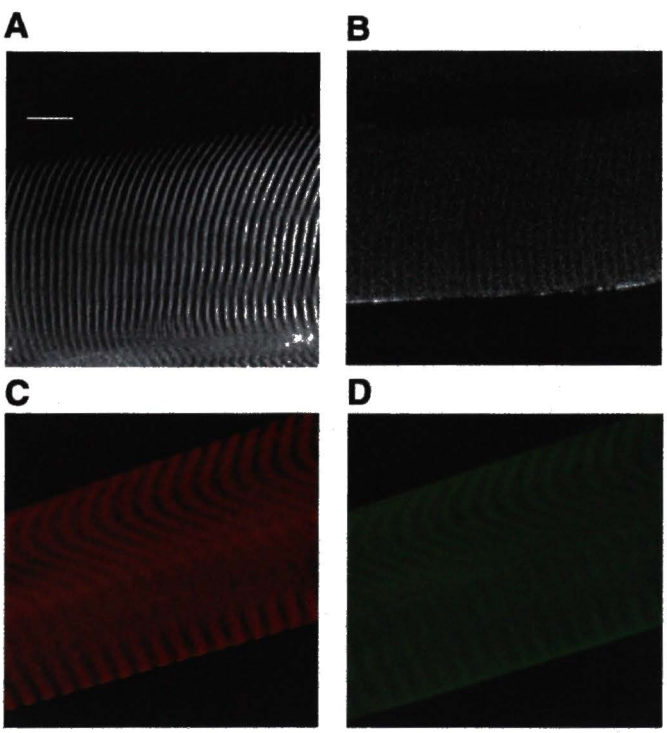

Figure 13: Localization of LC1 and RLC in skeletal muscle fibers. The LC1 (A and C) was labeled with 5'-IATR and exchanged as described in Fig. 8. The RLC (B and D) was labeled with 5'-IAF and exchanged by incubation in rigor solution at 30°C for 30 minutes. The striated pattern was better seen in slowly stretched fibers (C and D). A sequential exchange of LC1 and RLC in the same fiber (C and D) did not change the fluorescence pattern.

The confocal microscopy of LC1 in myofibrils (Fig. 12) showed that it predominantly binds to the central region of the A-band. Some binding was also observed in the Z-line. Incubation at lower temperature, without TFP, in rigor solution or the use of LC1 isolated from rabbit skeletal muscles did not affect the fluorescence pattern (not shown).

In contrast to myofibrils, after exchange of LC1 into muscle fibers the fluorescence was observed from the entire A-band and no fluorescence was in the Z-line (Fig. 13, A and B). The striated pattern was seen better in fibers stretched by slow pulling to produce 4-6 μm sarcomeres (Fig. 13, C and D)). A sequential exchange of LC1 and RLC in the same fiber did not affect the pattern (Fig. 13, C and D)). However, exchange of the RLC showed that it also binds to the I-band. I conclude that RLC does bind to fiber with about three-fold lower specificity than ELC.

d. Mobility of the C-terminus of LC1

i. Steady-state fluorescence anisotropy of LC1 in vitro

ATP had similar effect on the mobility of the C-terminus of LC1 when it was incorporated either in free S1 or in muscle fibers. The steady-state anisotropies of free S1(LC1) are shown in Table 3. The average r in the absence of ATP was 0.195±0.001. This is larger than the average r of free LC1 (r=0.166).

Addition of 4 molar excess of F-actin caused only a small increase in r, in spite of the fact that binding to much larger F-actin causes significant immobilization of the HC (Mendelson *et al.*, 1973). This suggests that the interaction of the HC with actin, and the direct

interaction of the LC1 with actin through the N-terminus, only weakly inhibits the motion of the C-terminus. Addition of ATP dramatically increased r. No such increase was seen with ADP or PP_i (not shown).

Table 3. Steady-state anisotropy of S1(LC1)

Sample	Anisotropy, r
S1(LC1)	0.195±0.001
S1(LC1)+A	0.216±0.009
S1(LC1)+ATP	0.233±0.002
S1(LC1)+A+ATP	0.233±0.001
S1(LC1)+ADP	0.198±0.001

Addition of ATP to acto-S1 increased r beyond the anisotropy characteristic of the acto-S1 complex, to the extent characteristic of S1+ATP (Table 3). Anisotropy of free LC1 was unchanged in the presence of either actin or ATP (data not shown). These results suggest that in the absence of ATP, the C-terminus of LC1 has considerable freedom of motion, and that it becomes strongly immobilized when ATP binds to the active site of S1.

ii. Time-resolved fluorescence anisotropy of LC1

Time-resolved measurements are summarized in Table 4. Anisotropy decays were obtained by the variable frequency phase-modulation fluorometry as previously described (Maliwal *et al.*, 1986; Cheung *et al.*, 1991). The data were fitted to the biexponential decay law $r(t)=r_o\Sigma g_i\exp(-t/\rho_i)$ where r_o is the limiting anisotropy in the absence of rotational motions, ρ is the rotational correlation time and g_i are the associated amplitudes. The results show that the two rotational correlation times were different for LC1 alone, S1(LC1), and S1(LC1) in the presence of ATP. In the presence of ATP the rotational

correlation times were the highest, indicating significant decrease in the mobility of the label on Cys178 of LC1. The average of the two rotational correlation times $<\rho>=g_1\rho_1+g_2\rho_2$ is 233 nsec. This is similar to the single correlation time (220-250 nsec) previously determined from time-domain data of fluorescence of dyes attached to the Cys707 of S1 heavy chain (Mendelson *et al.*, 1973).

Table 4. Anisotropy (r), lifetimes (τ) and rotational correlation times (ρ) of S1(LC1). Actin (A) was added in 4 molar excess over S1.

Sample	τ_1, τ_2 (nsec)	ρ_1, ρ_2 (nsec)
S1(LC1)	2.95, 0.55	66.6, 0.68
S1(LC1)+A		
S1(LC1)+ATP	3.02, 0.69	314.8, 1.45
S1(LC1)+A+ATP		
S1(LC1)+ADP		

Addition of ATP did not change τ_1 or τ_2, but increased ρ. The lifetimes and correlation times of free LC1 were unchanged in the presence of either actin or ATP (not shown). This confirms the results of steady-state measurements suggesting that the motion of the C-terminus of LC1 is strongly inhibited in the presence of ATP. Cheung *et al.* (1991) also reported two rotational correlation times (288.7 and 9.5 nsec) for 1,5-IAEDANS attached to Cys707 of S1. In this work the effect of ATP on the mobility of the fluorescent dyes attached to Cys707 of S1 and Cys178 of LC1 was opposite: ATP increased the mobility of dye on Cys707 and decreased the mobility of the dye on Cys178.

iii. Immobilization of the dye by LC1

The anisotropies of free rhodamine, free LC1 labeled at the C-terminus and of LC1 incorporated into the HC of S1 were 0.065, 0.166

and 0.195, respectively. The rotational correlation times of LC1 and of LC1 incorporated into S1 were 11.73 and 66.6 nsec, respectively. ATP affected neither the anisotropy nor correlation times of LC1.

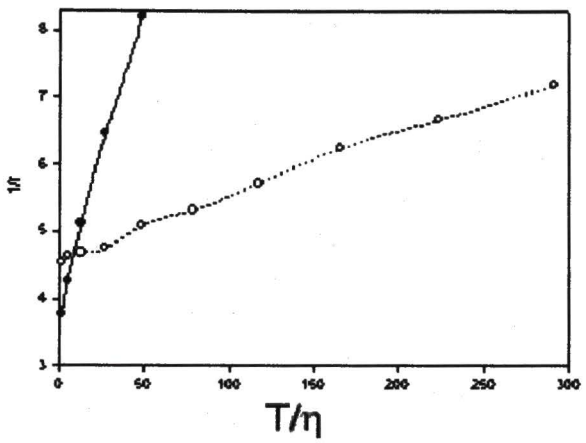

Figure 14: Dependence of LC1 fluorescence anisotropy on the viscosity of the solvent. Anisotropy [r] of 1 μM LC1 (open circles) and free 5'-IATR (filled circles) at various concentrations of glycerol was measured and the corresponding viscosities [η] of glycerol-water solutions were defined by a standard table (Sheely, 1932).

By measuring the dependence of anisotropy on the viscosity of the solvent (Perrin plot, Fig. 14), it was possible to estimate the "wobble" angle, α, through which the fluorophore rotates during the lifetime of the excited state (Lakowicz, 1986). This angle depends on the rigidity of the attachment of the dye to LC1 and on the segmental motion of the peptide. If $\alpha=0^0$, there is no rotation; if $\alpha=\pi/2$ the dye rotates freely. For LC1 labeled near the C-terminus α was around 30^o (Fig. 14). Assuming that rhodamine binds rigidly to the LC, this result suggests that the C-terminus of LC1 has greater mobility than the rest of the chain. This is consistent with the results of Huang *et al.* (1998).

iv. Fluorescence anisotropy of LC1 in muscle fibers

In muscle, Δr was measured from 5 fibers exchanged with fluorescent LC1. Since in rigor $r_\perp \approx r_\parallel$, the distribution of labels cannot be represented by a simple model[1]. The model-independent values Δr are summarized in Table 5.

Table 5. Anisotropies of muscle fibers carrying rhodamine labeled LC1.

Sample, state	$r_\perp$	$r_\parallel$	$\Delta r = r_\perp - r_\parallel$
LC1, rigor	0.183±0.018	0.174±0.024	-.009
LC1, relax	0.143±0.012	0.173±0.014	0.030

The fact that for rigor fibers Δr was small and that it was not statistically significantly different from 0 (t=0.736, P=0.595) suggests that the C-terminus of LC1 is disordered. This disorganization most likely results from the high mobility of the C-terminus. In relaxation, Δr was statistically different from 0 indicating a high degree of order. The preferred orientation was perpendicular to the fiber axis ($r_\parallel < r_\perp$). Because in the presence of ATP the C-terminus of free S1 was immobile, it can be suggested that the order is due to the immobilization. The difference between rigor and relaxation [$D = \Delta r(\text{relax}) - \Delta r(\text{rigor}) = 0.039$] is relatively large. This should be compared with the value of D=-0.221 obtained in a control experiment (consistent with earlier reports (Andreev *et al.*, 1993; Ajtai *et al.*, 1992; Berger *et al.*, 1996)), in which muscle myosin heavy chain was labeled

[1] For example, consider a simple Gaussian model. Θ defines the polar angle that a long axis of S1 makes with the vertical. Chromophores are distributed with a Gaussian probability around the mean value Θ_o with a standard deviation δ, i.e. the probability of dipole orientation at any angle Θ is given by $r(\Theta) = \exp[-(\Theta-\Theta_o)^2/2\delta^2]$. The angles Θ and δ cannot be estimated for LC1 in rigor by the method of Xiao *et al.* (1995).

at Cys707. Results obtained in muscle are consistent with the *in vitro* measurements and indicate that the C-terminus of the LC1 has considerable freedom of movement in rigor, but that it becomes immobilized and ordered in a relaxed muscle.

d. Mobility of the N-terminus of LC1

i. Binding of the N-terminus of LC1m to F-actin

LC1 binds to actin through the N-terminus (Andreev *et al.*, 1999; Timson *et al.*, 1998; Sweeney, 1995). A control experiment was carried out to confirm that the N-terminal extension of rhodamine labeled LC1m is able to bind to F-actin. S1(LC1m) was titrated with increasing amounts of F-actin and the fluorescence anisotropy was measured after every addition (Fig. 15). The anisotropy gradually increased and reached maximum when F-actin was in excess over S1, suggesting that the N-terminus of LC1m becomes immobilized.

The tip of the N-terminus of LC1 contains a number of lysine residues (Frank and Weeds, 1974) and it can be cleaved by trypsin. Chemical EDC cross-linking of S1(LC1m) with F-actin followed by limited trypsinolysis was conducted to check whether the N-terminus of exchanged LC1m binds to actin.

Figure 16 shows that both non-cross-linked and cross-linked S1(LC1m) were completely degraded by trypsin (lanes 1-4). After cross-linking of S1(LC1m) with F-actin the following fluorescent bands, indicative of LC1m, were detected: 21 kDa, 66 kDa, 120 kDa, 185 kDa and 235 kDa (Fig. 16, lane 5; Andreev and Borejdo, 1995).

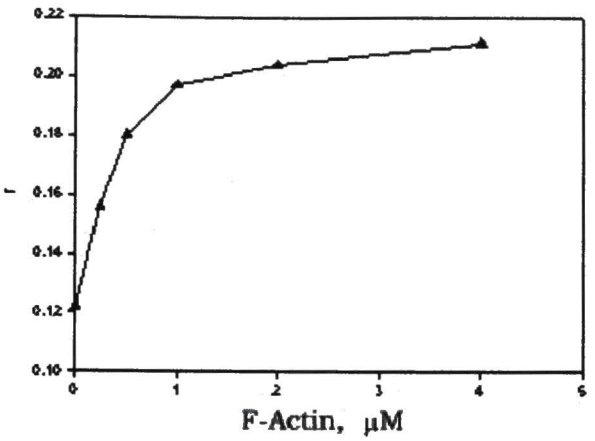

Figure 15: Dependence of anisotropy on the molar ratio of S1(LC1m) to F-actin. Increasing amount of F-actin was added to 1 mM S1(LC1m) in rigor solution (50 mM KCl, 10 mM Tris-HCl, pH 7.5) and the fluorescence anisotropy of rhodamine attached to LC1m was measured.

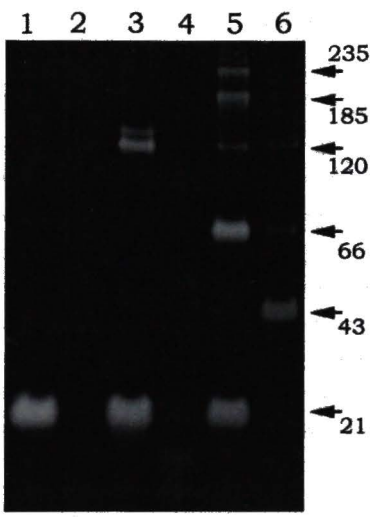

Figure 16: EDC cross-linking and trypsin digestion of S1(LC1m)-actin complex. The LC1m was labeled with rhodamine and exchanged into S1. The four molar excess of F-actin was added to S1(LC1m) and cross-linking was carried out with 75 mM EDC for 40 min at room temperature in 50 mM KCl, 0.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5. The samples were treated with 50 μg/ml trypsin. After 20 minutes the reaction was stopped by addition of 100 μg/ml soybean trypsin inhibitor and the samples were analyzed on 8% SDS-PAGE. Lane 1 – fluorescent LC1m of S1(LC1m). 2 – S1(LC1m) treated with trypsin. 3 – EDC cross-linked S1(LC1m). 4 - EDC cross-linked S1(LC1m) treated with trypsin. 5 –S1(LC1m) and F-actin cross-linked with EDC. 6 - S1(LC1m) and F-actin cross-linked with EDC and treated with trypsin.

After trypsin digestion, a new 43 kDa fluorescent band formed (Fig. 16, lane 6), which overlapped with the actin band on a Coomassie blue stained gel (not shown). This result confirms that the end of N-terminal extension of LC1m binds to F-actin.

ii. Steady-state fluorescence anisotropy of LC1m in vitro

ATP had similar effect on the mobility of the N-terminus of LC1 when it was incorporated either in free S1 or in muscle fibers. The average r of free S1(LC1m) in the absence of ATP was 0.168±0.002 (Table 6).

Table 6. Steady-state anisotropy of S1(LC1m).

Sample	R	% change from S1(LC1m) alone
S1(LC1m)	0.168±0.002	-
S1(LC1m)+A	0.273±0.002	62.5
S1(LC1m)+ATP	0.212±0.001	26.2
S1(LC1m)+A+ATP	0.223±0.001	32.7
S1(LC1m)+ATP+A	0.224±0.001	33.3
S1(LC1m)+ADP	0.176±0.001	4.0
S1(LC1m)+ADP+A	0.198±0.003	17.8

Addition of 4 molar excess of F-actin caused a large increase. However, addition of ATP to S1(LC1m) alone, also caused a significant increase in r (from 0.168 to 0.212). Addition of ATP to acto-S1 decreased r to the extent characteristic of S1+ATP. ADP and PP_i caused no change. The average r of free LC1m was unchanged in the presence of either actin or ATP (data not shown).

These results suggest that the N-terminus is immobilized by actin in the absence of ATP, and remains partially immobilized in the presence of ATP. The question arises whether this partial

immobilization is due to the N-terminus binding to actin even in the presence of ATP. It is shown below that this is not the case.

iii. Fluorescence anisotropy of LC1m in muscle fibers

In muscle, Δr was measured from 10 different fibers exchanged with fluorescent LC1m (Table 7). The average Δr in rigor (0.080±0.033) and in relaxation (0.037±0.004) were both significantly different from 0. These results suggest that, like in LC1 incorporated into free S1, the N-terminus of the LC1 is restrained in rigor, and that it remains restrained in a relaxed muscle.

Table 7. Anisotropy of muscle fibers carrying rhodamine labeled LC1m.

Sample, state	$r_{\perp}$	$r_{\parallel}$	$\Delta r=r_{\perp}-r_{\parallel}$
LC1m, rigor	0.184±0.002	0.264±0.001	0.080
LC1m, relax	0.186±0.002	0.223±0.002	0.037

e. Does LC1 bind to actin in the presence of ATP?

The acto-LC1 bond involves ionic forces (Andreev and Borejdo, 1995). It is therefore possible that adding ATP to rigor muscle dissociates only the HC, but that the whole S1 remains tethered to filaments by LC1. The N-terminus could then be immobilized by actin and not by ATP. Numerous ultracentrifugation and light scattering data suggest that the dissociation constant is large (Margossian and Lowey, 1978; Martson and Weber, 1975; Greene and Eisenberg, 1980). However, the actin-LC1 bond is fragile and the above experiments were carried out applying centrifugal or shear forces. To

check whether the actin-LC1 bond is retained in the presence of ATP and in the absence of shear forces, the control experiments were carried out.

i. LC1 cannot be cross-linked to F-actin in the presence of ATP:

If LC1 of S1(LC1) could bind to F-actin in the presence of ATP, then it should be possible to capture the LC1+actin complex by cross-linking. The LC1+actin complex migrates at 66 kDa. It is produced only when actin is in molar excess of S1 (Andreev and Borejdo, 1995).

Figure 17 compares cross-linking in the presence and absence of ATP. A 4 molar excess of actin was preactivated with EDC and mixed with S1(LC1) and S1(LC3). The solution was not stirred. One sample contained MgATP. The disappearance of ATP was monitored by a change in the turbidity of acto-S1 complex (Van Dijk *et al.*, 1998). As expected, Coomassie Blue staining (A) of the rigor S1(LC1)+actin complex (lane 2) showed a 66 kDa band. In addition, cross-linking both S1(LC1) and S1(LC3) produced 210 and 150/160 kDa bands characteristic of A+A+HC and A+HC/HC+A complexes (Andreev *et al.*, 1993).

When the reaction was carried out in the presence of ATP, no complex corresponding 66 kDa was obtained. Western blots using antibodies against HC (B) show that HC does not form a complex with actin. Consistent with earlier work (Van Dijk *et al.*, 1998) there was small amount 210 kDa complex. The pattern of cross-linking of the HC was the same for S1(LC1) and S1(LC3) (left and right panels of Fig.

17), suggesting that binding of LC1 to actin does not disturb HC binding.

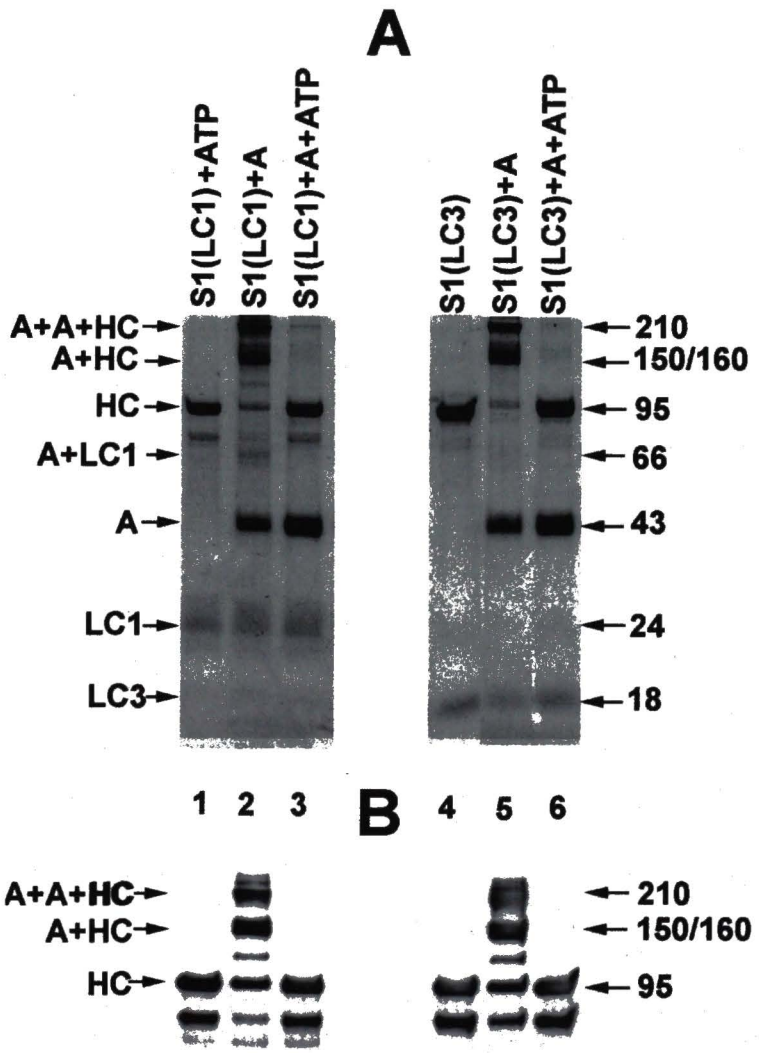

Figure 17: The effect of ATP on cross-linking of isoforms of S1 with actin. Actin was preactivated with 20 mM NHS and 10 mM EDC at 4°C. The reaction was stopped with 0.1 M β-mercaptoethanol after 20 min. Aliquots of preactivated actin were added to S1 or to S1 containing 5 mM MgATP and allowed to equilibrate for 30 min at 4°C. A: Gels stained with Coomassie Blue. Lane 1: 1 μM S1(LC1)+ 5 mM ATP, lane 2: 1 μM S1(LC1)+ 4 μM actin; lane 3: 1 μM S1(LC1)+ 4 μM actin + 5 mM ATP; lane 4: 1 μM S1(LC3)+ 5 mM ATP, lane 5: 1 μM S1(LC3) + 4 μM actin; lane 6: 1 μM S1(LC3)+ 4 μM actin + 5 mM ATP. B: Western blots stained with antibodies against myosin heavy chain. Lane assignments as in A.

ii. S1(LC1) and S1(LC3) dissociate at the same rate from the thin filaments.

The rate of ATP-induced dissociation of fluorescent S1(LC1) and S1(LC3) from myofibrils was compared. To carry out such an experiment quantitatively, the fluorophores must be resistant to photobleaching. Therefore, Alexa dye was used (Haugland, 1996). Alexa is a maleimide derivative, which makes labeling of Cys707 of the HC not feasible, because it renders the HC insensitive to dissociation by ATP (Pemrick and Weber, 1976).

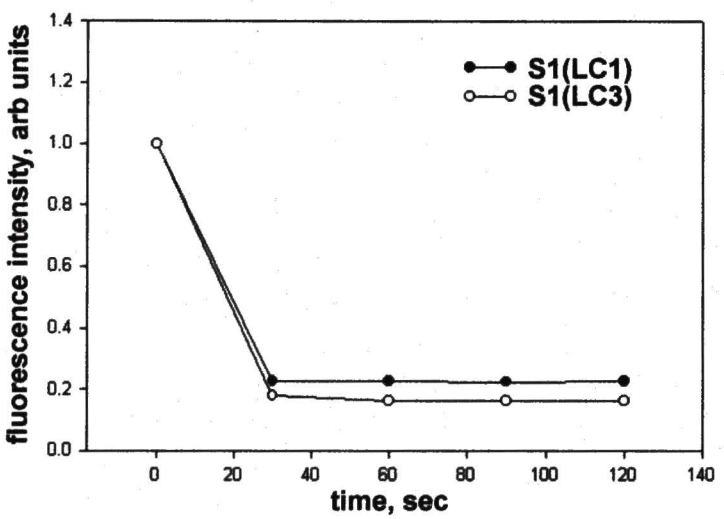

Figure 18: The rate of dissociation of S1(LC1) (filled cirlces) and S1(LC3) (open circles) from myofibrils. ELCs were labeled with Alexa by incubating with 5 molar excess of the dye at 0^0C for 3 hours, followed by dialysis and passage through Sephadex G-50 column. Labeled ELCs were exchange with the endogenous ELCs as described in Methods. The myofibrils were irrigated with S1(LC1) or S1(LC3) in EDTA-rigor solution (10 mM Tris-Acetate, pH 7.5, 50 mM KCl, 5 mM EDTA).

By this reason, isolated ELCs were labeled with Alexa dye, exchanged with endogenous ELCs of S1 and diffused into myofibrils in EDTA-rigor. To assess the extent of dissociation, EDTA-rigor was

gently replaced with relaxing solution and the fluorescence intensity of the myofibril was measured every 30 sec. Figure 18 shows that both S1(LC1) and S1(LC3) dissociated from myofibrils to a similar extent. This result again suggests that ATP is able to break the actin-LC1 complex.

CHAPTER FOUR

DISCUSSION

a. ADP promotes reorientation of S1 on F-actin

The process of ATP hydrolysis by myosin subfragment-1 has a number of intermediate states, with release of the products (ADP and γ-phosphate) being a rate-limiting step (Lymn and Taylor, 1971). It is believed that during this step the S1 undergoes a gross conformational change, which results in the swinging of the molecule on F-actin and the subsequent displacement of actin filament (Huxley and Simmons, 1971). It has been recently suggested that the RD of S1 is responsible for the mechanical events, while the role of the MD and the actin-binding interface has been diminished, if not excluded (Rayment *et al.*, 1993b). On the other hand, new data have appeared suggesting that it is the MD that could be responsible for the production of force, and the rotation of the RD is a mere consequence of it (Andreev *et al.*, 1993; Berger *et al.*, 1996; Burghardt *et al.*, 1997).

We investigated the role of MD using chemical cross-linking approach. Cross-linking with a zero-length carbodiimide reagent EDC is a sensitive method of assessing changes in proximity (Grabarek and Gergely, 1990). Even a small shift in the position of the amino- relative to the carboxyl-groups of the two moieties is expected to modify the rate of the reaction.

The finding that ADP had no effect on skeletal S1 when actin was fully or sparsely decorated with S1, but that there was a

significant effect at intermediate saturations can be explained by a "two state model" which was proposed earlier (Andreev *et al.*, 1993). In this model the heavy chain of S1 binds to one actin monomer (state 1) when actin filament is saturated with heads, and binds in a different orientation to two monomers (state 2) when it is unsaturated.

The S1 is known to cross-link to actin via two regions: Tyr626-Glu647 loop or Lys567-His578 (Sutoh, 1983). These regions are shown as red and yellow spacefill peptides on Figure 19, respectively.

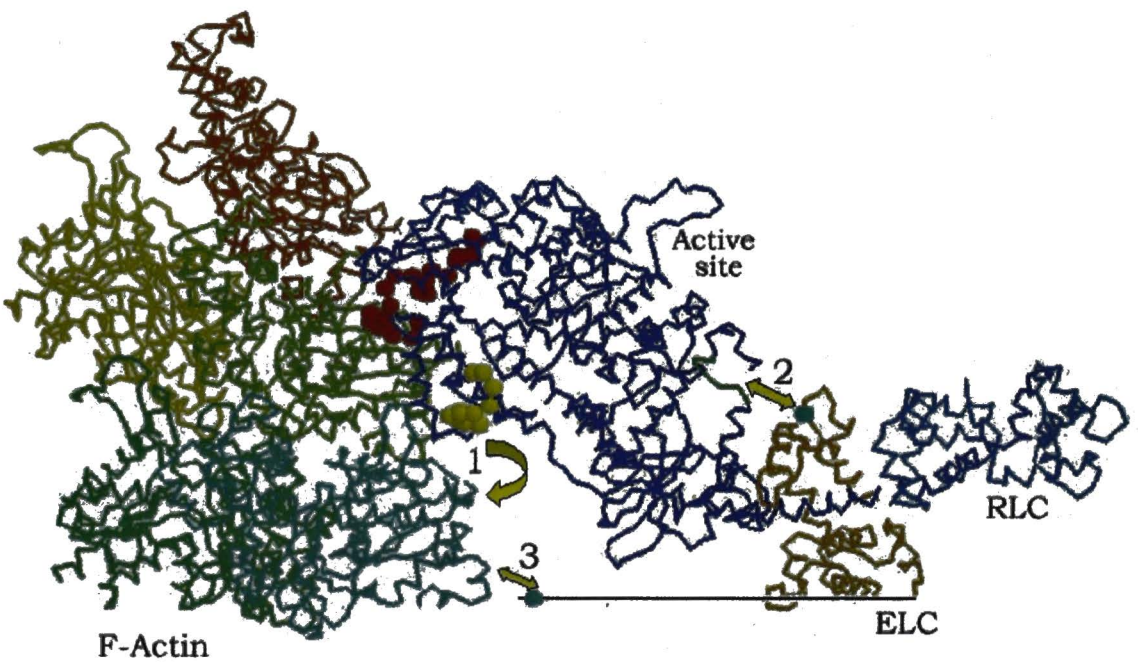

Figure 19: Conformational transitions in S1. Based on the refined structure of actin-S1 rigor complex (Morris and Mendelson, 1997). 1 - S1 cross-links to F-actin first through Tyr626-Glu647 (red spacefill) and then to Lys567-His578 (yellow spacefill). ADP promotes reorientation of S1 on F-actin. 2 – following the binding of ATP to the active sites, cys178 of ELC (cyan spacefill) binds to the Arg18-Arg24 loop of the HC (green). 3 – binding of ATP to the active site completely dissociates the N-terminus of ELC (cyan spacefill, drawn schematically). Five actin monomers are shown in different colors.

At intermediate ratios of S1 to actin ADP increased the formation of 160 and 210 kDa complexes (Table 1). In the 160 kDa

complex S1 cross-links to actin through Lys567-His578, and in 210 kDa complex it also cross-links to a second actin through Tyr626-Glu647 (Sutoh, 1983; Andreeva *et al.*, 1993). On the other hand, the formation of 150 kDa complex, in which S1 is cross-linked to actin only through Tyr626-Glu647 site, was not affected by ADP. Formation of 160 kDa and 210 kDa complexes in rigor is increased much higher with decrease in the molar ratio of S1 to actin, than formation of 150 kDa complex. Combining these evidences, it can be concluded that S1 binds to F-actin first through the Tyr626-Glu647 cross-linking region and then reorients and also binds through the Lys567-His578 cross-linking region.

The following model explains this process. The actin filament can be viewed as a long single row of monomers "A" and S1 molecule can bind to the filament in two possible states. In state 1 it cross-links to one actin monomer through Tyr626-Glu647 and in state 2 it reorients and cross-links to two actin monomers through both cross-linking regions (Fig. 19, arrow 1).

Adsorption of S1 proceeds as two consecutive, reversible reactions

$$A + S1 \leftrightarrow A{\cdot}S1$$

$$A{\cdot}S1 \{+ A\} \leftrightarrow A{\cdot}S1{\cdot}A$$

The first reaction is a "classical" second-order reaction where S1 adsorbs to F-actin from the solution. The second reaction is an isomerization where S1 changes its conformation and now binds to two actins. Brackets {} are used to emphasize the fact that S1 now

attaches to the second actin. In this reaction, the rate constants do not depend on the absolute concentration of actin. They depend only on the probability that the actin, neighboring to the point of attachment of S1, is free (Andreev *et al.*, 1993).

Let us assume that S1 first adsorbs from solution in the state 1 (A·S1). After initial binding S1 changes orientation and, providing that the neighboring binding site is free, binds additional actin monomer (A·S1·A). The kinetic constants of these reactions are k_{+1}, k_{-1} and k_{+2}, k_{-2}, respectively. $K_1 = k_{+1}/k_{-1}$ is the equilibrium adsorption constant for state 1 (dimension $S1^{-1}$). $K_2 = k_{+2}/k_{-2}$ is the equilibrium constant of transition from state 1 to state 2 (dimensionless).

The model makes the following predictions: at high molar ratios S1:actin, most S1s are in state 1. Moderate decrease in the equilibrium constant K_1 with no concurrent change in the constant K_2, causes only a small decrease in the number of S1s in this state. Consequently, the number of S1s in state 2 is not significantly changed. At low degrees of saturation, most S1s are bound in state 2 (there is a few S1s in state 1). Moderate decrease in the equilibrium constant K_1 causes a small decrease in the number of S1s in already sparsely populated state 1, and consequently only a small increase in S1s in state 2. However, at intermediate saturations, the release of some S1s leads to a situation where many actin sites adjoining to the remaining S1s in state 1 become vacant with the result that some S1s in state 1 can now undergo transition to state 2. Consequently, the total number of S1s in state 1 remains approximately unchanged while the total number of S1s in state 2 is significantly increased.

ADP is an analog, which decreases K_1 by a moderate amount (Highsmith, 1976; Trybus and Taylor, 1982; Duong and Reisler, 1987a, 1987b). However, if an analog decreases the equilibrium constant K_1 by a large amount, as AMP-PNP or PP_i do (Highsmith, 1976; Trybus and Taylor, 1982; Duong and Reisler, 1987a), the model predicts that the number of S1s in state 1 always decreases. The behavior of S1s in state 2 depends on the molar ratio S1/actin: at high ratios where most S1s are in state 1, there is a significant decrease in the number of S1s in state 2. Consequently, many actin sites adjoining to the remaining S1s in state 1 become vacant. The result is that the remaining S1s in state 1 are now able to undergo transition to state 2 and the total number of S1s in state 2 is significantly increased. At low and intermediate degrees of saturation, on the other hand, the large release of S1s leaves only a few S1s in state 1 able to undergo transition to state 2 to replace those S1s which slowly dissociate. The result is that the number of S1s in state 2 decreases.

It is worth pointing out that the well known effect of ADP that causes the reorientation of rhodamine dipole bound to Cys707 of the heavy chain in skeletal muscle fibers (Borejdo *et al.*, 1982; Tanner *et al.*, 1992) may have the same origin as the one described here. The thin filaments in muscle are intermediately saturated with heads (the ratio of myosin heads to actin in vertebrate skeletal muscle varies between 1:3.2 (Morimoto and Harrington, 1974) to 1:1.8 (Squire, 1981)). Therefore, it is possible that adding ADP to skeletal muscle causes a mild dissociation and redistribution of the heads between states 1 and 2.

The effect of ADP on the orientation of S1 is in agreement with the recent electron microscopy studies, which showed that ADP changes orientation of smooth muscle S1 and brush-border myosin I by 30-35^0 with respect to F-actin (Jontes *et al.*, 1995; Whittaker *et al.*, 1995). On the other hand, the fact that when the ratio of S1:actin was large, ADP had no effect on cross-linking is consistent with other works, in which an excess of S1 was used and no effect of ADP was detected on the conformation (Gollub *et al.*, 1996; Borejdo *et al.*, 1997; Getz *et al.*, 1998; Smyczynski and Kasprzak, 1997).

b. Conformation of the C-terminus of LC1

Similarly to EDC cross-linking, the anisotropy of fluorescence is a sensitive probe of orientation (Morales, 1984). In the absence of ATP, the anisotropy r and rotational correlation time ρ of free S1(LC1) are small (Table 4). The C-terminus does not rotate completely freely. The rotational correlation time of free LC1 was ρ=11.73 nsec and increased to 66.6 nsec upon binding of LC1 to the HC. Values of r, ρ and Δr increased significantly upon addition of ATP. Addition of ATP to acto-S1 increased ρ to the level characteristic of the whole S1 (Mendelson *et al.*, 1973). ATP did not alter the excited state lifetime showing that the change is not due to the environment of the probe. This strongly suggests that immobilization was a result of binding of the C-terminus to the HC (Fig. 19, arrow 2). In rigor fibers, the disorder of the C-terminus of LC1 is large, in spite of the fact that the N-terminus and the catalytic domain of S1 are bound to actin.

The transition from rigor to relaxation results in the imposition of a significant degree of order on the C-terminus (Table 5). This is consistent with the immobilization of the C-terminus observed *in vitro*.

Paradoxically, the catalytic domain of S1 retains little order in relaxation (Thomas and Cooke, 1980; Borejdo *et al.*, 1982). The order of the C-terminus of LC1 can be explained by the fact that the orientation of the RD in relaxation is imposed by the thick filaments (Davis and Harrington, 1987; Harrington *et al.*, 1988). The RD is separated from the MD by a flexible joint (Highsmith *et al.*, 1979; Adhikari *et al.*, 1997). Thus in relaxed fibers, the motor domain of S1 is disordered, but the regulatory domain is ordered.

c. Conformation of the N-terminus of LC1

In the absence of ATP, r of free S1(LC1m) is small (Table 6). Addition of an excess of actin significantly increased r. This is undoubtedly due to the binding of the N-terminus to actin (Prince *et al.*, 1979; Andreev *et al.*, 1999). The binding occurs only when actin is in excess (Andreev and Borejdo, 1995) and takes place through the N-terminal lysines and the N-terminal α-amino group (Andreev *et al.*, 1999; Timson *et al.*, 1999). The molar ratio S1:A was 1:4. In spite of the fact that binding to F-actin immobilizes the N-terminus, it is clear that ATP does so also. The addition of ATP to S1 (no actin) increased r over 26%, but the addition of actin to S1 containing ATP increased the anisotropy only a little. ATP had the same effect on muscle myosin. In fibers, the disorder in rigor was small. In muscles actin is in excess over myosin (Morimoto and Harrington, 1974; Squire, 1981). Therefore, a large Δr in rigor is not unexpected because the N-

terminus binds to thin filaments. Indeed, it has been recently shown that LC1 binds to thin filaments in myofibrils (Andreev and Borejdo, 1999).

Addition of ATP increased disorder (Fig. 19, arrow 3), but the N-terminus was not completely mobile in spite of the fact that the control experiments showed that ATP dissociates LC1 from F-actin. It can be concluded that the anisotropy of the N-terminus remained high due to the immobilization by ATP.

d. Implications for myosin conformational dynamics

The effect of ADP on the EDC cross-linking of S1 to F-actin unequivocally supports the two-state theory proposed earlier (Andreev *et al.*, 1993). According to it, S1 binds first to one actin monomer and then reorients and binds to two actin monomers. The sites of EDC cross-linking on S1 are located in the 50 kDa portion of S1 and the 50/20 kDa loop (Sutoh, 1983). However, it must be taken into account that the primary actin binding site of S1 is hydrophobic in nature and located in the 50 kDa portion (Rayment *et al.*, 1993b). Recently, it has been shown that the Glu506-Lys561 peptide of S1 could be cross-linked to F-actin by an aromatic residue cross-linker (Bertrand *et al.*, 1997). Moreover, the pattern of cross-linking in the presence of ATP and its analogs was different from the pattern in the presence of ADP or in rigor. This further strengthens the conclusion that the change in acto-S1 interface occurs upon the release of the products of ATP hydrolysis. This suggests that the MD of myosin head may contribute to the power stroke.

The results show that binding of ATP to the active site on the MD has also a significant effect on the mobility of the essential light chain. This is in spite of the fact that the MD and the RD are separated by a large distance (Rayment *et al.*, 1993a; Highsmith and Eden, 1993) (the distance between the α carbons of Cys178 on LC1 and Trp131 on HC is 36.9 Å). A related observation was made earlier (Marsh and Lowey, 1980; Marsh *et al.*, 1982). In those experiments, the rate of fluorescence change of the fluorophore attached to Cys178 was equal to the rate of ATP hydrolysis (as evidenced by the change of fluorescence of ATP-sensitive Trp). Thus the events occurring at the active site were simultaneously felt at Cys178 of LC1 and at the ATP-sensitive Trp in the HC (probably residue 440 (Reshetnyak *et al.*, 1999). This observation is fully consistent with results presented here.

The simplest explanation of changes in the mobility is that ATP hydrolysis causes the swing of the RD, consistent with earlier suggestions (Rayment *et al.*, 1993b). This swing induces conformational change in LC1. The small β-sheet on which Cys178 is located (Cys178–Met145) forms a cleft with a loop Arg18–Arg24 on the 27 kDa portion of the heavy chain. The immobilization can occur because ATP closes this cleft. The small β-sheet probably binds to the positively charged loop on the 27 kDa domain of HC (Fig. 17, arrow 3). The analysis of thermal B-factors of atoms and amino acids in the PDB structure of skeletal myosin subfragment-1 (PDB entry 2MYS, RD (Rayment *et al.*, 1993a)) confirms this idea (Borejdo *et al.*, submitted). The most likely reason why ATP causes this cleft to close, is that the nucleotide binding at the active site results in a rotation of the RD.

Since binding and hydrolysis of ATP cause the power stroke, these results suggest that at least part of the power stroke involves rotation of the RD. However, at present the possibility can not be excluded that ATP causes the Arg18–Arg24 loop on the 27 kDa fragment to change conformation and move toward LC1 (or that LC1 moves closer to HC) without rotation of the RD. In smooth muscle S1, the binding of ATP causes negatively charged G-helix of ELC (Arp96-Ile138) to bind to the positively charged 25/50 kDa loop of the HC (Met140-Tyr261). In rigor, the F-helix of ELC binds to the N-terminal part of the HC (Gln21-Asn29).

The above results suggest that the power stroke is associated with the conformational changes in both the MD and RD.

CHAPTER FIVE

CONCLUSION

The effect of ADP on binding of S1 to F-actin confirms a previously proposed two-state hypothesis that S1 first binds to one actin monomer and then reorients and binds to two actin monomers. The result suggests that the conformational changes in the MD may contribute to the power stroke.

Binding of ATP to the active site of myosin induces immobilization of the C-terminus of LC1. This immobilization is most probably due to the binding of the Cys178-Met145 β-sheet of LC1 and the Arg18-Arg24 loop of the HC, resulting from the rotation of the RD.

Binding of ATP to the active site completely dissociates the N-terminus of LC1 from F-actin. However, the N-terminus is partially immobilized by S1 in the presence of ATP.

From the presented evidences, it can be concluded that both the changes in the actin-myosin interface and the swing of the lever arm can contribute to the power stroke.

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