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Variable surface loops and myosin activity: Accessories to a motor

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Abstract

The catalytic head of myosin is a globular structure that has historically been divided into three segments of 25, 50, and 20 kDa. The solvent-exposed, proteolytically-sensitive surface loops of myosin that join these three segments are highly variable in their sequences. While surface loops have not traditionally been thought to affect enzymatic activities, these loops lie near the ATP and actin-binding sites and have been implicated in the modulation of myosin's kinetic activities. In this work we review the wealth of data regarding the loops that has accumulated over the years and discuss the roles of the loops in contributing to the different activities displayed by different myosin isoforms.

Myosin is a molecular motor that uses the energy of ATP to power its movement relative to actin filaments. Present in all eukaryotic cells, it is fundamental to such processes as cell division, vesicle transport, and muscular contraction. The most studied form of myosin is myosin II (conventional myosin), which is found in skeletal muscle, cardiac muscle, smooth muscle, and nonmuscle cells, but there are also many classes of unconventional myosins that vary considerably in their tail regions and carry out different roles in the cell (Sellers and Goodson, 1995). To date, at least 15 classes have been identified, indicating that myosins have diverged considerably for specialized roles in the cell (Bement *et al*., 1994; Cope, 1996; Mermall *et al*., 1998). Myosins hydrolyze ATP and bind actin in a cyclical fashion in order to produce movement relative to actin filaments, as shown in the Scheme I below (Lymn and Taylor, 1971):

Scheme I.

Myosin binds very tightly to actin in the absence of nucleotide, but dissociates from actin (k_{10}) upon binding ATP (k_5); the ATP is hydrolyzed by myosin while in this weakly-bound state (k_2). In the absence of actin, phosphate release (k_3) is very slow and is the rate-limiting step of the ATPase cycle. Phosphate is induced to dissociate upon reassociation of actin in a strongly-bound state (k_{11}, k_7) and thus actin is said to activate the ATPase activity through this acceleration of phosphate release. It is thought that the force-generating stroke occurs following phosphate release (reviewed in Goldman, 1987). The stroke is immediately followed by ADP release (k_8), which allows ATP to bind once again (k_5) and dissociate the myosin from the actin (k_{10}). In the presence of actin a conformational change involving the weak-to-strong actin-binding transition (an isomerization simplified here as k_{11}), rather than the P_i release step itself (k_7), is rate-limiting (Geeves, 1991).

Myosin II is a hetero-hexamer of two heavy chains joined in a coiled-coil tail, with two pairs of essential and regulatory light chains attached to the neck region. In the cell, multiple myosin IIs bind in the tail region to form bipolar thick filaments, which allows the heads to pull actin filaments together. The heavy chain includes the catalytic domain, which binds actin and ATP and is responsible for the molecule's motor activity. The catalytic domain with the light chain-binding region is known as subfragment-1 or S1. S1 has historically been divided into three domains generated by the tryptic cleavage of the motor into segments of 25, 50, and 20 kDa (shown in green, red and white, and blue in Figure 1). The crystal structure of chicken skeletal S1 (Rayment *et al*., 1993) suggests that rather than functioning as separate domains, these segments work together to propagate changes from the nucleotide binding pocket and the actin binding face to the light chain binding region, which then may act as a lever arm to produce the working stroke. The linkers of the three domains, the 25–50 K and 50–20 K loops, were not resolved in the crystal structure, suggesting that they adopt multiple conformations, and are located on the surface of the molecule, explaining their proteolytic accessibility.

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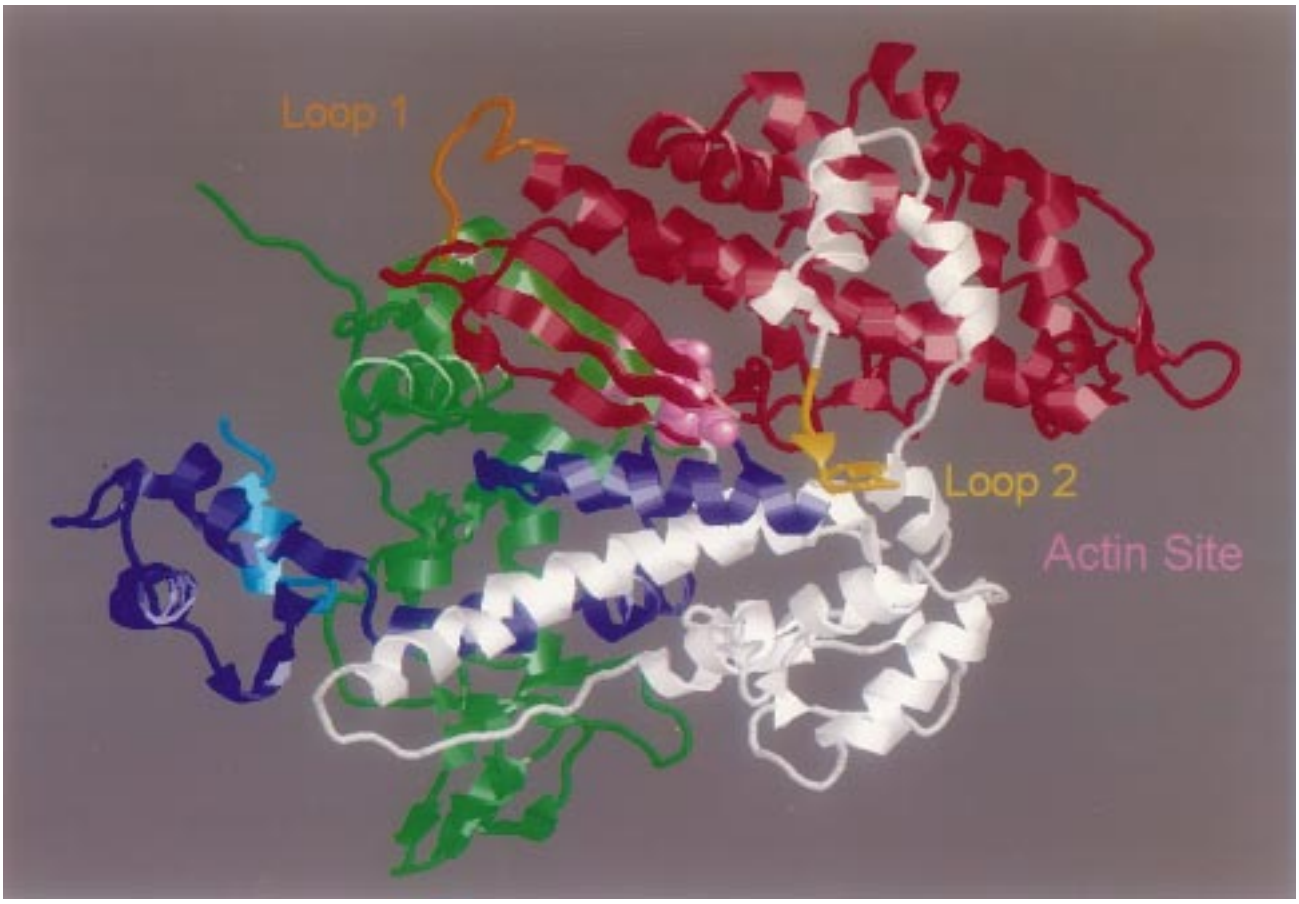


Fig. 1. Structure of Myosin II Subfragment 1 (Rayment *et al*., 1993). The 25, 50, and 20 kDa "domains" are shown in green, red and white (upper and lower 50 K domains), and blue. Loop 1 (orange) connects the 25 and 50 kDa domains and lies near the ATP binding pocket, while the 50–20 kDa loop, Loop 2 (yellow), lies in the actin binding face. The light chain binding region is part of the 20 kDa domain (blue) and is an α-helical extension that was truncated to solve the *Dictyostelium* crystal structures (Gulick *et al*., 1997). Nucleotide is shown in magenta (spacefill) and residues close to the site of truncation are shown in cyan. (Figure courtesy of Dietmar Manstein.)

Despite the fact that the loops are highly variable in sequence, and thus may have been considered to be functionally unimportant, they have long been the focus of myosin research because of their intriguing locations: the 25–50 K loop (Loop 1) is situated near the nucleotide binding site, and the 50–20 K loop (Loop 2) lies in the actin binding face, as shown by cross-linking and proteolytic protection studies done prior to the solving of the S1 crystal structure. Additionally, the loops are hotspots for alternative splicing in several forms of myosin.

In this paper we review the research published on the two loops, including proteolysis, crosslinking, alternative splicing, evolutionary, chimeric, and structural studies, in an effort to characterize the role of these highly variable surface loops in myosin function.

Proteolysis and crosslinking studies

Skeletal S1 can be tryptically cleaved into three segments of 25–27, 50, and 20 kDa (Balint *et al*., 1978). It was subsequently shown that S1 cleavage inhibits actin activated ATPase activity (Mornet *et al*., 1979) and decreases S1's affinity for actin (Yamamoto and Sekine, 1979). The 25–50 K and 50–20 K cleavage sites could be protected by addition of ATP and actin (Mocz *et al*., 1984; Mornet *et al*., 1981), and were mapped to the ATP binding region (Okamoto and Yount, 1983; Szilagyi *et al*., 1979) and the actin binding face (Mornet *et al*., 1981; Sutoh, 1982, 1983), respectively. Crosslinking of myosin to actin followed by proteolysis and protein sequencing showed that a lysine-rich segment on myosin is the cleavage site and binds to the acidic *N*-terminus of actin (Yamamoto, 1989, 1990). Additionally, it was shown that the binding of actin and myosin must be different in this region in the presence and absence of ATP, suggesting that the loop might play a role in the transition from weak to strong binding (Yamamoto, 1989). The addition of negatively charged residues to the *N*-terminus of actin increased the V_{max} of actin-activated ATPase activity without affecting motility, again suggesting that the interaction between these charged segments is responsible for the weak to strong transition (Cook *et al*., 1993). Smooth muscle myosin S1 cleavage at a lysine-rich site is also blocked by actin binding, as well as by a conformation known as the 10S folded state (Ikebe and Hartshorne, 1986); subsequent study suggested that this site is involved in the weak binding state (Ikebe *et al*., 1993).

Cleavage of Loop 1 does not affect the actin-activated ATPase activity of S1 (Bobkov *et al*., 1996; Chaussepied

and Morales, 1988; Mornet *et al*., 1979, 1981). Ohishi *et al*. (1993) suggested that an activation of motility of tryptically-cleaved chymotryptic S1 could be attributed to a reduction of drag force due to a lowered affinity for actin; however, the motility of S1 with individually-cleaved loops was not examined (both loops were cleaved). Bobkov *et al*. (1996) studied the ATPase activity and motility of selectively digested HMM. This study showed that isolated cleavage of Loop 1, but not of Loop 2, caused a reduction in motility. Loop 2 cleavage does severely reduce actin affinity (as reflected in an increased K_m of the actin-activated ATPase activity), causing the filaments to diffuse away in the absence of methylcellulose, but the velocity was unaffected. Conversely, cleavage of Loop 1 alone had no effect on the K_m of the ATPase activity. Thus, proteolysis studies suggest that Loop 2 cleavage affects the actin-activated ATPase activity, while Loop 1 cleavage affects motility.

Alternative splicing and isoform differences

Many types of myosin are alternatively spliced and are differentially expressed in various tissues. Among these are vertebrate cardiac, smooth muscle, and neuronal nonmuscle myosins, *Xenopus* nonmuscle myosin, *Drosophila* nonmuscle myosin (*zipper*), carp skeletal myosin, and scallop skeletal and catch muscle myosins. A surprising number of these isoform differences appear to be focused in the loops. One exception to this general pattern is the alternative splicing of *Mhc*, the *Drosophila* muscle myosin heavy chain gene, which is discussed below.

Cardiac myosin

The mammalian α and β cardiac myosins are 93% identical in their amino acid sequences (McNally *et al*., 1989) (Figure 2), but display about twofold differences in their kinetic activities: the α isoform has an *in vitro* velocity of 5 μm/s and has an actin-activated ATPase activity of 6 s^{-1}, while the β isoform moves actin filaments at about 2 μm/s and has an ATPase activity of about 3 s^{-1} (VanBuren *et al*., 1995). The differences in sequence between the two isoforms appear largely in the two loops (Goodson *et al*., 1999). Studies of Loop 2 chimeric constructs with the cardiac myosin loops (described below) suggest that the differences in actin-activated ATPase activity are due primarily to changes in Loop 2 (Uyeda *et al*., 1994).

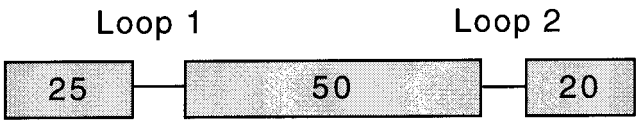
Smooth muscle myosin

Vertebrate smooth muscle myosin II isoforms are derived from a single gene that is alternatively spliced in two regions (Babij *et al*., 1991). No major enzymatic differences have been found to result from alternative splicing in the *C*-terminal region. However, there is a distinct expression pattern of the *N*-terminally-spliced isoforms: the intestinal isoform differs from the vascular and uterine isoforms by a seven amino acid insertion at the 25–50 K junction (Babij, 1993; Hamada *et al*., 1990; White *et al*., 1993). Intestinal tissue is a phasic fast-contracting muscle, while the latter two are tonic muscles that contract and maintain tension for long periods of time, and it is probable that the shortening speed differences arise from this alternative splicing. Kelley *et al*. (1993) showed that the isoforms are tissue-specific and that the intestinal isoform has higher *in vitro* motility (0.75 μm/s vs 0.3 μm/s) and actin-activated ATPase activities (gizzard myosin's V_{max} is twice that of uterine's[1]). They ruled out the possibility that these differences are due to the presence of different light chain isoforms through light chain exchange experiments. However, other sequence differences in the head-rod junction or in Loop 2 could not be ruled out as the cause of enzymatic differences.

To eliminate this ambiguity, Rovner *et al*. (1997) expressed homogeneous populations of smooth muscle HMM that varied only in the presence or absence of the seven amino acid insertion. The segment was inserted into the normally non-inserted uterus form, and was removed from the intestinal isoform; the activities of all four HMMs were then compared. In both pairs, the presence of the insert increased the enzymatic activities of the isoform (ATPase rate and *in vitro* velocity). While the insert did affect the actin-activated ATPase activity, it did not seem to completely determine the activity. In contrast, adding the insert conferred upon the uterine isoform the velocity of the intestinal isoform. That is, even when the uterine isoform contains the seven amino acid insert, its K_m in the ATPase assay is still too high to be measured at 100 μM actin, while the intestinal (gizzard) isoform has a K_m of about 30 μM whether the insert is present or not. The V_{max} also appears to be lower for the two uterine HMMs, but the authors point out that the lack of saturation makes it impossible to fit the data to determine a V_{max} or K_m. It seems that the 7-amino acid Loop 1 insert is capable of determining the *in vitro* velocity of the myosin and can also affect the actin-activated ATPase activity, but this rate is ultimately controlled by another part of the molecule.

The recent publication of the smooth muscle myosin structure (see below) highlights the possible interaction between Loop 1 and the essential light chain (Dominguez *et al*., 1998). The actin-bound head of smooth muscle myosin has been shown to tilt in relation to the long axis of the actin filament upon ADP release (Gollub *et al*., 1996; Poole *et al*., 1997; Whittaker *et al*., 1995), and a strain-dependent mechanism for ADP release from smooth muscle myosin has been proposed (Cremo and Geeves, 1998). Perhaps Loop 1 serves some role in the regulation of this process.

[1]Actin-activated ATPase V_{max}s and K_ms are quoted from the papers being reviewed; the data were not graphed in the paper for the reader to judge goodness of fit or extent of saturation. However, most assays were done in the 5–50 μM actin range.



Loop 1

Nonmuscle myosin II

chicken, human MHC-B	DHNIP	GELER
chicken MHC-B1a	DHNIP**PESPKPVKHQ**	GELER
human MHC-B1a	DHNIP**QESPKPVKHQ**	GELER
Xenopus MHC-B1b	DHNIP**TESPKAIKHQSGSLLY**	GELER

Smooth muscle myosin II

chicken MHC (aorta)	DTSIT	GELEK
chicken MCH (intestinal)	DTSIT**QGPSFSY**	GELEK

MyoK (*Dictyostelium*) TSVSPNNSSGGGIGGSGGGNGGIPQYDGGSDDRP-
SPPMGRGMGMPGMVGRGGLPTRGGGPPSRGGGPPPTRGRGGPPPPIPQNRGAPPP-
VSNGGAPPPPVARGPVAPPPTRGAPPTRGGGPANRGGRGGGPPPVSTSRGGGGY-
GGSSK

MyoJ (*Dictyostelium*) FAAMGNMIKESTSSSSINGINTSSDGICVTPPP-
SPMKKSPVDKSVEE

Loop 2

Nonmuscle myosin II

chicken, human MHC-B	ELWKD	VDRIV
chicken MHC-B2	ELWKD**EIQNIQRACFYDNITGLHDPP**VDRIV	
human MHC-B2	ELWKD**EIQNIQRASFYDSVSGLHEPP**VDRIV	

Carp skeletal myosin II

10°C isoform	SALKVLALLY VAVP EAEAAGKKG GKKKGGSF
intermediate isoform	SALKVLALLY VAVP E EGGGKKA GKKKGGSF
30°C isoform	SSLKVLAFLY ATHGAEAEGGGGKK GKKKGGSF
(chicken skeletal:	SSVKTLALLF ATYGGEAEGGGGKKGGKKKGSSF)

Rat cardiac myosin II

alpha	SSLKLMATLFSTYASADTGDSGKGKGGKKKGSSF
beta	SSLKLLSNLFANYAGADAPV DKGKGKAKKGSSF

myr5 (rat Class IX) SSYVRQLIGMDPVAVFRWAVLRAAIRAMAVLRE-
AGRLRAERAEKAEAGVSSPVTRSHVEELPRGANTPSEKLYRDLHNQIIKSLKGLPWQG-
EDPRRLLQSLSRLQKPRTFFLKSKGIKQKQIIPKNLLDSKSLRLIISMTLHDRTTKSL-
LHLHKKKKPPSISAQF

Fig. 2. Sequences of inserts at Loop 1 and Loop 2 (from McNally *et al.*, 1989; Reinhard *et al.*, 1995; Sellers and Goodson, 1995; Yazu *et al.*, 1999). Nonmuscle and smooth muscle myosin IIs, as well as unconventional myosins, display inserts at the two loops (bold type). The underlined serines in the nonmuscle myosins are potential phosphorylation sites (Pato *et al.*, 1996). MyoK (Yazu *et al.*, 1999), MyoJ (Reinhard *et al.*, 1995), and myr5 (Reinhard *et al.*, 1995) have large loops. The sequence of carp skeletal Loop 2 varies with temperature (Reinhard *et al.*, 1995).

Nonmuscle myosin

Human and avian. Vertebrate nonmuscle myosin II is encoded by two genes, MHC-A and MCH-B, that are 87% identical in the head but only 72% identical in the rod region (Takahashi *et al*., 1992) (Figure 2). Among other sites, MHC-A and -B differ in the length and sequence of the loops. MHC-B is alternatively spliced not only in Loop 1, with tissue-specific inserts of 10 and 16 amino acids (Bhatia-Dey *et al*., 1993; Itoh and Adelstein,1995; Pato *et al*., 1996), but also in Loop 2, with an insert of 21 residues (Itoh and Adelstein, 1995) (Figure 2). Interestingly, a colon adenocarcinoma cell line, Caco-2Bbe, contains MHC-A with an 11-amino acid insert at Loop 1 (Bement *et al*., 1994). The ten amino acid insert in MHC-B's Loop 1 has been found in human and avian tissues, and differs only in the most *N*-terminal residue; the 16 residue insert found in human tissue differs from the constitutively inserted *Xenopus* nonmuscle form (see below) in only three amino acids (Bhatia-Dey *et al*., 1993; Itoh and Adelstein, 1995). All of the Loop 1 inserts contain the Ser-Pro-Lys consensus sequence for proline-directed kinases, such as cyclin-p34 cdc2 kinase (Pato *et al*., 1996). It is not yet known for which kinase the nonmuscle myosin is a substrate. The actin-activated ATPase and motility rates of baculovirally-expressed chicken nonmuscle HMM II-B with and without the 10 amino acid insert were examined (Pato *et al*., 1996). The inserted form displayed a slight increase in actin-activated V_{max} and K_m[1] and a 20% increase in velocity. Incomplete (30–40%) phosphorylation of the inserted form further increased the ATPase activity, resulting in a final increase in activity of about 2-fold over the non-inserted form's activity (0.47 s^{-1} vs 0.2–0.28 s^{-1}). Phosphorylation was described as having no significant effect on the sliding velocity of one preparation (40% phosphorylation). The authors suggest that the modest increases in *in vitro* activity may indicate that this insert may serve as a localization domain or in a function other than to increase enzymatic activities, with phosphorylation acting to regulate association (Pato *et al*., 1996). It would be interesting to know the *in vitro* velocity as well as the localization pattern of the fully phosphorylated myosin, and the signal transduction pathways that result in phosphorylation of the inserted form.

Human and avian brains express the different isoforms in a tissue- and developmentally-dependent manner (Itoh and Adelstein, 1995). While most tissues do not express the inserted isoforms, MHC-B with the 10-amino acid insert is the major MHC-B isoform in human cerebrum and retina, while the 21-amino acid Loop 2 insertion is present in human cerebrum and cerebellum tissue. Neuronal cell lines show reversible insertions at both Loop 1 and Loop 2 in response to growth factor and butyrate stimulation, suggesting that the inserted forms play a role in growth and differentiation. PC-12 cells treated with nerve growth factor develop neurite outgrowths, and the expression of the 10-amino acid inserted MHC-B form appears simultaneously; removal of NGF results in disappearance of both the neurites and the insert (Itoh and Adelstein, 1995). Other cell lines also show a correlation between cell differentiation and expression of the inserted MHC-B isoform.

Xenopus. The predominant *Xenopus laevis* nonmuscle myosin II form is MHC-A. MHC-B is totally absent from some tissues, such as kidney or skeletal muscle (Bhatia-Dey *et al*., 1993). As in the case of human and avian MHC described above, the MHC-A and -B sequences of *Xenopus* are highly identical in the head but diverge in the loop regions and the S1-S2 hinge region. *Xenopus* MHC-B constitutively contains the 16 amino acid insert in Loop 1 (Kelley *et al*., 1996). MHC-A and -B differ in their subcellular localizations and enzymatic activities; MHC-A has about three times the velocity and actin-activated V_{max} as MHC-B, but a similar K_m for actin[1] (Kelley *et al*., 1996). MHC-B is found in the periphery of the cells and may be involved in leading edge extension or retraction, while MHC-A is found in the cytoplasm and at the mitotic spindle (Kelley *et al*., 1996). *Xenopus* MHC-B can be phosphorylated by Cdc2 kinase, and Ser214 of MHC-B, which lies in the inserted sequence, is phosphorylated during maturation of oocytes, but not in G2 interphase, suggesting that there is tight regulation of cortical reorganization through the activity of the phosphorylation of MHC-B (Kelley *et al*., 1995).

Drosophila melanogaster. The single *Drosophila* nonmuscle myosin II heavy chain gene, *zipper*, is involved in cytokinesis, cellularization, axon patterning, and tissue development (Kiehart *et al*., 1989; Young *et al*., 1993). Like other nonmuscle myosins, it is alternatively spliced at Loop 1, inserting a 40 amino acid segment (Mansfield *et al*., 1996). PCR of the splice junctions in *Drosophila simulans* (five million years removed evolutionarily from *D. melanogaster*) identify an insertion of 120 nucleotides with only two nucleotide differences from the *D. melanogaster* sequence (Mansfield *et al*., 1996). The conservation of this exon suggests that the insert has some as yet unidentified biological importance.

Skeletal myosin

While muscle fibers have been well characterized and skeletal myosin has been used for years in biochemical studies, sequence data has been less forthcoming from these systems. It is known that different isoforms are expressed in different mammalian fiber types and at different stages of development (reviewed in Schiaffino and Reggiani, 1994). *Rana pipiens* muscle fibers are also commonly studied, and as in mammalian systems, different fiber types express different myosin isoforms, as defined by tail sequence (Lutz *et al*., 1998). It will be interesting to see whether the loops differ between isoforms and play a role in determining their activities.

Carp (Cyprinus carpio). Certain fish acclimate to temperature changes and change cruising speed shortly thereafter (Fry and Hart, 1948). The myofibrillar Mg^{2+} ATPase activity was found to be changed after goldfish and other teleosts acclimate from warm to cold temperatures (Heap *et al*., 1985; Johnston *et al*., 1975); acclimation to low temperature results in higher ATPase activity, allowing maintenance of constant physiological and biochemical rate processes despite temperature variation. Carp, like goldfish, acclimate to temperature variations by changing their myofibrillar activity; these changes have been attributed to changes in myosin isoform expression (Gerlach *et al*., 1990). Myosin isolated from fast skeletal muscle in carp acclimated at 10°C moves actin filaments at a higher velocity than does myosin from 30°C acclimated carp, when assayed at the same temperature (Chaen *et al*., 1996). The Mg^{2+}-ATPase activities of myosin and S1 from the 10°C carp are 2–4 times higher than the activities of the 30°C isoforms (Hwang *et al*., 1991), and more force and work is generated by muscle fibers from cold-acclimated fish (Altringham and Johnston, 1985). Analysis of the sequence of myosin heavy chain from carp acclimated at 10°C, 20°C, and 30°C shows that in addition to changes in the rod that contribute to thermal stability (Imai *et al*., 1997), there were changes in the *N*-terminal domain, Loop 1, and Loop 2 (Hirayama and Watabe, 1997). A study of 29 distinct PCR clones from a carp genomic library showed that carp encode nine different Loop 2 sequences and they are most closely related to the Loop 2's of higher vertebrate skeletal myosin (Kikuchi *et al*., 1999) (Figure 2). Thus, the differences in catalytic activities of the three temperature isoforms seem to be in large part due to differences in the two loops, and carp express different loop isoforms to acclimate to different temperatures.

Scallop. An additional example of alternative splicing at the loops to create myosins with different functions comes from molluscs. Scallops have both striated (phasic) and smooth (tonic) muscles, but in *Argopecten* both muscle heavy-chain isoforms are produced through alternative splicing of a single gene at four exons (Nyitray *et al*., 1994). *Placopecten* striated and catch muscle myosins are also thought to be alternatively spliced products of a single gene, differing in five small areas (Perreault-Micale *et al*., 1996). Comparison of the *Placopecten* striated and catch muscle sequences shows that they are 97% identical, and the *Argopecten* and *Placopecten* S1 sequences are 94% identical (Perreault-Micale *et al*., 1996). The only region in which all four MHCs vary is exon 5, which includes the 25-50 K junction (Loop 1) and differs in 18 amino acids, including one residue of the ATP-binding P-loop. The other exons that are alternatively spliced between the catch and striated forms include part of the actin binding site, a region following Loop 2, the hinge-rod junction (which is also a site of alternative splicing in *Drosophila* muscle myosin and differs in MHC-A and -B nonmuscle myosin), and a non-helical part of the tail. Papain digestion of the scallop isoforms yields S1, which eliminates the latter two alternatively spliced regions. The *Placopecten* striated S1 actin-activated ATPase V_{max} is 2–3 times that of the catch S1's V_{max}, with a similar K_m. The *in vitro* motility of the *Placopecten* isoforms were 0.85 and 3.8 μm/s for catch and striated, respectively; *Argopecten* striated isoform moved at 2.6 μm/s (Kurzawa-Goertz *et al*., 1998). Perreault-Micale *et al*. (1996) postulate that changes in Loop 1 are responsible for the differences in enzymatic activities between the two species.

A transient kinetic study of S1s derived from the *Argopecten* striated muscle, *Placopecten* striated muscle, and *Placopecten* catch muscle (Kurzawa-Goertz *et al*., 1998) was undertaken to establish which steps of the actin-myosin cycle differ between the types of myosins. The striated S1s were similar in all of their measured rates (nucleotide binding to S1 and actin-S1, actin binding rates, affinity for actin, and affinity for ADP), with none of the measured rates differing by more than a factor of two. The kinetics of most steps were similar for the catch muscle S1, as well. However, this isoform showed a much higher affinity for ADP (78 μM) when bound to actin than did either of the striated S1s (670 and 480 μM). Because the myosins are so similar to one another (97% identity between isoforms of the same species) except in exons 5 and 13, the authors suggest that the differences in Loop 1 may cause the changes in ADP affinity. Because there are also changes in the ATPase activity, Loop 1 might also be responsible for changes in the rate-limiting step of ATPase activity, possibly phosphate release. However, it is also possible that the differences in exon 13, which lies between the actin and ATP sites, affect both of these activities.

An exception to the rule: the Drosophila myosin heavy chain gene

There are many examples of alternative splicing at the loops to generate myosins with varied activities, including the *D. melanogaster* nonmuscle myosin gene *zipper*. The single muscle heavy chain gene (*Mhc*) of the same species, however, presents an entirely different story: only four regions of the head are alternatively spliced (Standiford *et al*., 1997), and both Loop 1 and Loop 2 are in unspliced regions (Bernstein and Milligan, 1997; George *et al*., 1989). The hinge-rod region encoded by exon 15a and b seems to be expressed in a tissue-specific manner and correlates with the velocity of muscle contraction (Collier *et al*., 1990), but the functions of the four alternatively spliced regions in the head are less obvious (Bernstein and Milligan, 1997). The region encoded by exon 7 (residues 301–335) includes five alternative residues near the ATP binding pocket and Switch I loop, and may affect nucleotide interactions (Bernstein and Milligan, 1997). Exon 3 encodes the *N*-terminal β-barrel that may be involved in head–head interactions; the regions encoded by exons 3 and 9 both

lie near the SH1-SH2 helix, which is thought to undergo large conformational changes (Patterson *et al*., 1997). Exon 9 encodes a region (amino acids 472–528) that seems to greatly affect myosin activities, and there are large differences in the contraction speed and tension levels generated by the different exon 9 spliceoforms. Finally, the exon 11-encoded region may interact with the differentially-expressed essential light chains. The regions encoded by exons 3, 9, and 11 converge on the fulcrum point (near the sulfhydryl helix) of the molecule and thus may greatly affect the activity at this point, which can then be amplified through the lever arm. It is interesting that the mode of alternative splicing to generate isoforms are so different between muscle and nonmuscle myosins (*zipper* myosin, see above) in the same organism.

While we have presented the splicing of the *Drosophila* myosin heavy chain as an exception, splicing to cause changes in the protein core to change activities is a commonly observed phenomenon. In contrast, many myosins display alternative splicing at the loops, under specific cellular and organismal conditions, indicating that these regions must carry out important roles in distinguishing the functions of myosin isoforms, and many of these isoforms have been shown to differ in their enzymatic activities. Additionally, these inserts have been conserved between species, indicating their importance.

Large loop inserts

Most myosins contain loops of 3–20 residues and in some cases are alternatively spliced to produce larger loops. Recently, however, unconventional myosins have been found that contain very large inserts in the loops. For example, myr5, a member of the class IX myosins, contains an insertion of 120 amino acids in Loop 2 (Figure 2) (Reinhard *et al*., 1995). The insert does not seem to block actin binding, because the myosin displayed normal ATP-regulated actin binding; it is possible that the insert somehow acts to regulate interactions with actin. An unconventional myosin cloned from *Dictyostelium*, MyoK, contains an insert of 142 amino acids in Loop 1 (Figure 2) (Yazu *et al*., 1999). MyoK is expected to be a member of the myosin I class, which contain ATP-insensitive actin binding regions; the high Gly, Pro, and Arg content of the insert suggests that this domain may serve as an actin binding region for MyoK. MyoJ, another unconventional myosin from *Dictyostelium*, has a 47 amino acid Loop 1 insert (Figure 2) (Hammer and Jung, 1996). Conventional nonmuscle myosin (*zipper*) in *D. melanogaster* also has an unusually large loop, 40 amino acids, as mentioned above (Mansfield *et al*., 1996). These inserts are large enough to be separate domains, capable of carrying out binding and enzymatic functions separate from the motor, as opposed to modifying the kinetics of the motor as many of the small, alternatively spliced loops do. It will be interesting to see what the functions of these domains are and how they contribute to the diversity of myosin activities.

Sequence studies of the loops

New members of the myosin superfamily of proteins are continually being identified (Mermall *et al*., 1998), and the comparison of these sequences has brought new insights into myosin structure-function relationships. Cope *et al*. combined phylogenetic and structural analysis to identify conserved sequences and to group myosins into 13 classes (Cope *et al*., 1996). While the bulk of this work focuses on the conserved residues, they do observe that there is some 'conserved variation' in the Loop 1 sequences, and note that myosins II, V, VIII, and XI display this type of 'signature' conservation in the loops.

Goodson *et al*. (1999) were specifically interested in the conservation of sequences in the loops. They hypothesized that if the loops are kinetically unimportant, they should show higher sequence variability than the rest of the molecule; if, on the other hand, the loops are important for function, then their sequence variation should be suppressed when myosins of like activities are compared. They showed that there is substantial suppression of variation in the loop regions for myosins of similar activities (Figure 3), including some suppression for myosin Vs and myosin Is in the Loop 1 region. These results suggest that there is evolutionary pressure to avoid changes in the loops, and therefore they are likely to play an important role kinetically. It should be noted that while other researchers had compared myosin sequences before, they had not grouped them according to kinetic function, and thus any conservation in the loops would not have been visible. Therefore, although the loops are generally considered to be highly variable, it seems that they are actually well conserved evolutionarily.

Chimeric studies

Loop 2

The idea that the loops carry out specific functions in the myosin cycle seemed possible from proteolytic, cross-linking, and alternative splicing data, and in 1994 it was proposed that Loop 2 may be largely responsible for setting the maximum actin-activated ATPase rate, while Loop 1 may control velocity through ADP release (Spudich, 1994). Earlier that year chimeras of *Dictyostelium* myosin with its Loop 2 replaced with the analogous loop from four other myosins with different enzymatic activities were found to have varying enzymatic activities (Uyeda *et al*., 1994). The chimeric myosins' ATPase activities correlated with the relative activities of the donor myosins, indicating that Loop 2 did indeed carry out an enzymatic role. It was also notable that several of the chimeras had ATPase activities higher than wild type's, demonstrating that it is possible to increase myosin's activities through changes in the loop. The velocities of the chimeras seemed unrelated to the origin of the loops. It should be noted that this was the first of many myosin loop

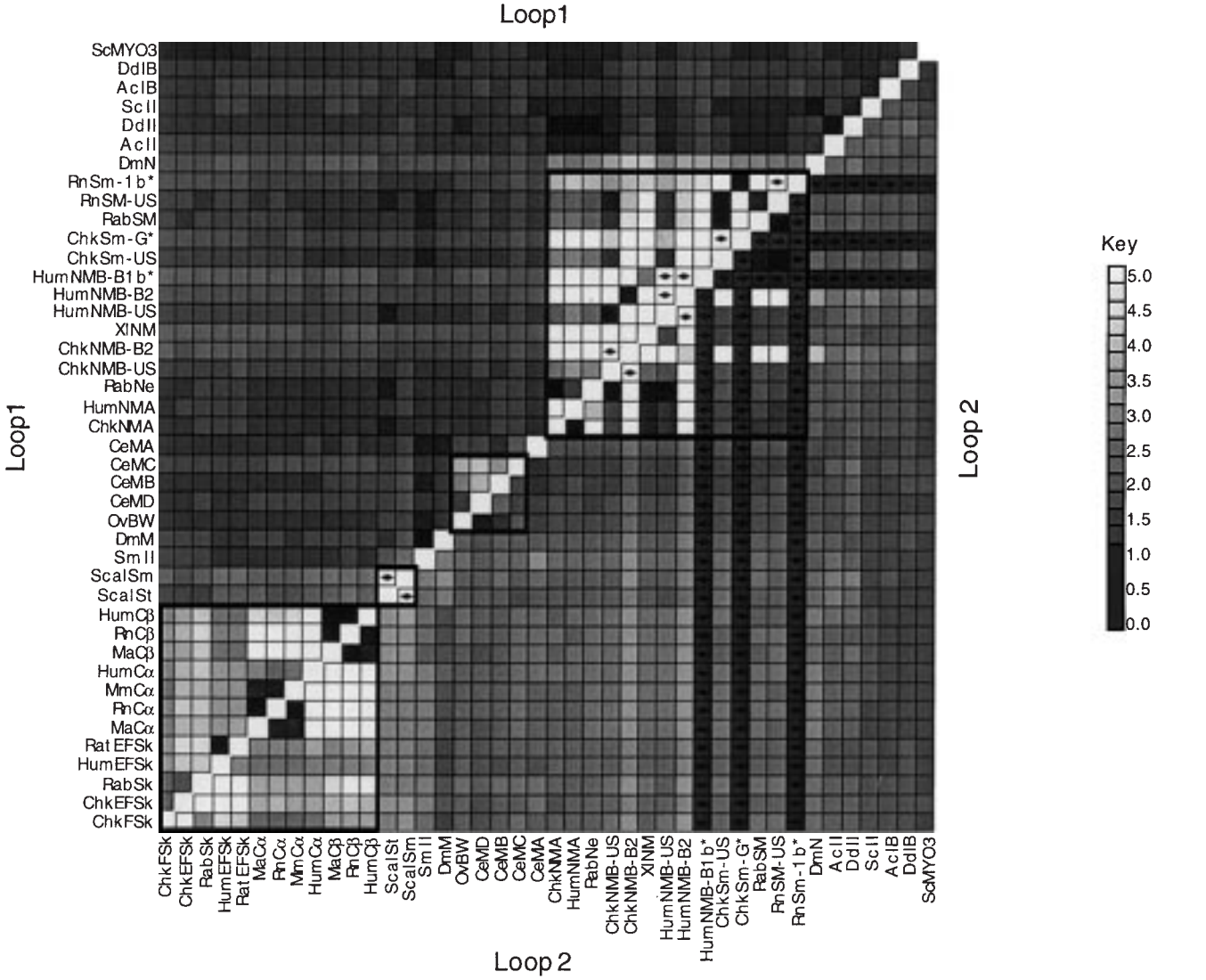


Fig. 3. Distance ratios of the loops of myosin (after Goodson *et al.*, 1999). The distance between sequences of the loop and the sequences of the myosin core of different myosins are compared as the ratio D_{loop}/D_{core}. A ratio of < 1 means that less change has occurred in the loop than in the rest of the protein, and thus the rate of change of the loop is considered to have been "suppressed" (Goodson *et al.*, 1999). The rates of change of the two loops are not identical, as shown by the different patterns on the two sides of the grid. Additionally, myosins of like activities (e.g., the cardiac β-myosins, cardiac α-myosins, skeletal myosins, and smooth myosins) show significant suppression (darker squares). Diamonds indicate splicing of the loop to create a second isoform without corresponding splicing of the other loop. Figure courtesy of H. Warrick. Accession numbers (or references) and abbreviations are as follows: *Acanthamoeba*: myosin IB (AcIB) P19706, myosin II (AcII) P05659; *C. elegans*: MYOA (CeMA) P12844, MYOB (CeMB) P02566, MYOC (CeMC) P12845, MYOD (CeMD) P02567; **Chicken**: embryonic fast skeletal (ChkEFSk) P02565, smooth muscle (ChkSM) P10587, smooth muscle splice form from gizzard (ChkSM-G) Kelley *et al.*, 1993, smooth muscle splice form from aorta (unspliced loop) (ChkSM-US) Kelley *et al.*, 1993, nonmuscle type A (ChkNMA) P14105, nonmuscle type B (ChkNMB) M93676, nonmuscle type B unspliced loop (ChkNMB-US), M93676, nonmuscle type B splice form B2 (ChkNMB-B2) M93676, adult skeletal (ChkSk) P13538; *Dictyostelium*: myosin II (DdII) A26655, myosin IB (DdIB) A33284; *Drosophila melanogaster*: muscle cDNA301 (DmM) A32491, nonmuscle (DmNM) A36014; **Human**: cardiac beta (HumCb) P12883, cardiac alpha (HumCa) D00943, embryonic fast skeletal (HumEFSk) P11055, nonmuscle type A (HumNMA) M81105, nonmuscle type B (HumNMB) M69181, nonmuscle type B unspliced loop form B (HumNMB-US) Takahashi *et al.*, 1992, nonmuscle type B splice form B2 (HumNMB-B2) Takahashi *et al.*, 1992, nonmuscle type B splice for B1b (HumNMB-B1b) Adelstein personal communication; *Mesocritus auratus* (**Golden hamster**): cardiac alpha (MaCa) L15351, cardiac beta (MaCb) L12104; **Mouse**: cardiac alpha (MmCa) M76598; *Onchocerca volvulus* (**a nematode**): body wall (OvBW) M74066; **Rabbit**: smooth muscle (RabSm) M77812, neuronal (RabNe) S21801, skeletal muscle (RabSk) A35557; **Rat**: cardiac beta (RnCb) P02564, cardiac alpha (RnCa) S06005, embryonic fast skeletal (RnEFSk) P12847, smooth muscle (RnSM) S61948, smooth muscle splice form 1A (unspliced) (RnSM-US) S61948, smooth muscle splice form 1B (RnSM-1B) S61948; **Scallop**: striated muscle (ScalSt) S13557, smooth muscle (ScalSM) Nyitray *et al.*, 1994; *Schistosoma mansonii*: myosin II (SmII) L01634; *Xenopus laevis*: nonmuscle (XlNM) A47297; *S. cerevisiae*: MYO1 (ScII) S12322, MYO3 (ScMYO3) Goodson *et al.*, 1995. Note: Human and chicken nonmuscle type B2 myosins contain both the Loop 1 and Loop 2 inserts, so it was unnecessary to include type B1, which contains only the insert in Loop 1. The Loop 2 sequence HumNMB-BIb is not known.

chimera studies, and did not answer every question associated with the loops. For example, assays were done under standard ATPase conditions, at 1 mg/mL (23 μM) actin. This concentration is subsaturating, and thus it was not clear whether Loop 2 primarily affected V_{max} or K_m. Certainly, it is easy to imagine that this

surface loop in the actin binding face could change the affinity for actin, while it is more difficult to envision how the loop could affect the rate-limiting conformational change that allows phosphate release.

Rovner *et al.* (1995) expressed, purified, and biochemically characterized smooth muscle HMM chimeras with cardiac and skeletal myosin Loop 2s. Smooth muscle myosin is highly regulated, with phosphorylation of the light chain activating phosphate release from the active site (Sellers, 1985). The primary observation of this chimera study is that replacement of the smooth muscle HMM Loop 2 abolishes light chain phosphorylation regulation of HMM activity. That is, the dephosphorylated form is normally inactive, but the chimeric myosins' phosphate release rate and motility are increased when compared with the dephosphorylated native myosin. The authors note that the phosphorylated chimeras have an increased affinity for actin relative to wild type and conclude that the loop is only capable of changing K_m, rather than having any effect on V_{max}. It should be noted, however, that the dephosphorylated (unregulated) forms show V_{max}s and K_ms that correlate with the activities of the donor myosins. The authors speculate that because the velocity of the phosphorylated myosins is decreased (native smooth muscle myosin > cardiac chimera > skeletal chimera), the loop may affect ADP release as well, but this was not tested directly.

Rovner went on to show that both the greater length and decreased charge in the 'lysine pocket' at the *C*-terminal end of the loop are necessary for regulation of smooth muscle HMM (Rovner, 1998). An additional 12 amino acids are conserved in regulated vertebrate myosins' Loop 2 *N*-terminus. In this careful study, constructs with and without this 12-amino acid sequence and the lysine-rich sequence at the *C*-terminus were tested for their activities in the phosphorylated and dephosphorylated forms. Because subtraction of the *N*-terminal 12 amino acids caused an increase in activity of both forms, while its addition to the skeletal loop caused a decrease in activity, it seems that this sequence may inhibit interaction with actin, thus allowing the molecule to be inactive in the dephosphorylated form. The subtraction of the *N*-terminal amino acids from the smooth muscle myosin loop as well as the increase in charge in the loop showed both a marked increase in V_{max} as well as decreased K_m in the actin-activated ATPase assay (Rovner, 1998).

The idea that Loop 2 can affect both K_m and V_{max} was further supported by studies of *Dictyostelium* chimeras in which the charge and length of the loop was systematically changed (Furch *et al.*, 1998) and in a follow-up study of the activities of Uyeda *et al.*'s substituted *Dictyostelium* chimeras (Murphy and Spudich, 1999). The addition of charge, but not length, to Loop 2 increased the affinity for actin as well as the V_{max} resulting in an overall 30–70-fold increase in catalytic efficiency (Furch *et al.*, 1998). Strikingly, the addition of 8 or 11 uncharged amino acids (variations of Gly-Asn-Asn) did not significantly change the activity, and small increases in charge also did not have much effect (Furch *et al.*, 1998). A later study using the loop with highly increased charge indicated that the greater V_{max} is due to increased interaction with the negatively charged region of actin subdomain 1 (Van Dijk *et al.*, 1999). As suggested by Furch *et al.*, the addition of charge in a random orientation seems to be less efficient in its activation of myosin ATPase activity than the addition of specific residues through substitution of natural Loop 2's: a difference in net charge of only +1 in the skeletal chimera Loop 2 (Murphy and Spudich, 1999) has an activity that is 6-fold greater than the activity of the synthetic +1 chimera. The studies also point out that Loop 2 has no effect on nucleotide binding (Furch *et al.*, 1998; Murphy and Spudich, 1999) or release (Murphy and Spudich, 1999) from S1. Additionally, Loop 2 was shown to affect actin binding both in the absence and presence of nucleotide, indicating a mechanism for Loop 2's effect on V_{max} (Furch *et al.*, 1998; Murphy and Spudich, 1999). Finally, the rate of actin-S1 dissociation by ATP (k_5, k_{10}) (Murphy and Spudich, 1999) was changed in a manner that reflected the affinity of the chimera for actin in its nucleotide-bound state. This could explain the slowed velocity of the chimeras in the Uyeda *et al.* (1994) study without invoking a change in ADP release rates; that is, if ATP-induced actin-myosin dissociation is slowed enough by changes in Loop 2, it may negatively affect velocity (see *Dictyostelium* Loop 1 chimeras below).

Complete deletion of Loop 2 has more severe effects on actin activation of ATPase activity and actin on-rates, resulting in a null phenotype *in vivo* in *Dictyostelium* and complete abrogation of *in vitro* motility (Knetsch *et al.*, 1999), without affecting the rate of mantATP binding to S1 or mantADP affinity for actoS1. Interestingly, this mutant displays an elevated basal ATPase rate (k_3), shows a higher ATP on-rate to actomyosin (k_5), and does not quench pyrene actin fluorescence, indicating that this conformation easily allows phosphate release and ATP binding, but does not change appropriately upon binding actin. However, actin binding is still able to weaken affinity for ADP to the same extent as for wild type. Thus, bringing the two ends of the loop together seems to have uncoupled phosphate release from productive actin interactions, indicating differences in communication paths for ADP and phosphate between actin and nucleotide binding sites (Knetsch *et al.*, 1999); these results suggest that Loop 2 is involved in the phosphate release pathway.

In summary, it appears that Loop 2 can affect the regulation of activation by light chain phosphorylation, actin affinity both in the presence and absence of nucleotide, and maximal activation of ATPase activity. It also seems that while charge increases can mimic the effects of Loop 2 changes, the residues of the naturally-occurring loops may be arranged in the order which produces the maximal efficiencies of the activities.

Loop 1

The relative average force and unitary force generated by smooth muscle HMM isoforms with and without the seven amino acid Loop 1 insert [examined earlier by Kelley *et al.* (1993) and Rovner *et al.* (1997)] were measured in the laser trap (Lauzon *et al.*, 1998). The presence of the insert decreased the t_{on} (the attachment time) but did not affect the displacement ($d = 10$ nm) or the relative average force. Thus, the addition of the insert changes the kinetics rather than the mechanics of the cycle. The constructs showed additional differences in t_{on} at lower ATP concentrations. Together these results indicate that both the ADP release from and ATP binding to actin-myosin are affected by the smooth muscle myosin insert at Loop 1, with the inserted form allowing faster detachment from actin as a result of the increases in rates at both steps. Of course, at saturating ATP concentrations, one would expect to see differences between the two only as a result of differences in ADP release rates. These results complement Rovner's earlier findings (Rovner *et al.*, 1997) that the insert affects motility and suggest that ADP release and ATP binding differences contribute to the differences in shortening velocities of the phasic and tonic muscles in which these isoforms are found.

Sweeney *et al.* (1998) also used baculovirus-expressed smooth muscle HMM to create a bank of Loop 1 chimeras, and measured the motility, ADP release, and ATPase activities of the chimeras. They found that motility correlates with ADP release rate, as predicted by Siemenkowski *et al.* (1985). More importantly, their results would suggest that ADP release rate correlates with loop length rather than with the activity of the donor myosin (i.e., loop sequence). Additionally they deleted the loop altogether (Δ25–50) and measured the hydrolysis rate, mantATP on-rate, mantADP on-rate, and mantADP release rate from actin-myosin. Δ25/50 showed changes not only in mantADP affinity and mantATP on-rate, but also in the rate of ATP hydrolysis. It is possible that when the two ends of the loop are brought together, the activity of the myosin could be negatively affected by structural changes in the nucleotide pocket, resulting in changes in all of these activities.

The results of the chimera experiments in this study seem quite straightforward: Loop 1 length, rather than sequence, determines myosin activity. However, none of the chimeras had even greater than one-half the ADP release activity of wild type, regardless of loop length; even the chimeras with longer loops than wild type had very low activities. All of the chimeras had decreased actin-activated ATPase V_{max} (ranging from 47–70% of wild type activity[1]) with wide variation in K_m; no other activities were measured. Thus, it is possible that the higher activity of the longer-looped constructs is merely due to a relief of some sort of inhibition specific to the chimeric constructs, rather than being instructive about the true role of the loop. When the *sequence* of the wild type construct was changed, while length was maintained, the activities changed: an alanine substitution, a charge reversal, and a conversion to the rabbit smooth sequence all showed decreases in activities. Additionally, a three-proline insertion caused a significant decrease in activity. The authors attribute these differences in activity to differences in flexibility of the loop. Overall, the data presented in this work support the hypothesis that the sequence, rather than length, of Loop 1 determines ADP release and subsequently velocity.

Another chimeric study used *Dictyostelium* myosin as the host with the Loop 1s from *Acanthamoeba* myosin II and skeletal myosin II (Murphy and Spudich, 1998). Skeletal myosin has higher *in vitro* velocity and ATPase activity than does *Dictyostelium* myosin; *Acanthamoeba* myosin was chosen because its sequence is closely related to *Dictyostelium* myosin's (Sellers and Goodson, 1995), yet its motility is almost an order of magnitude lower (Ganguly *et al.*, 1992). The two chimeras were able to complement a myosin II null mutant, indicating normal *in vivo* behavior: the chimera-transformed *Dictyostelium* cells were capable of growing well in suspension and developed normally into spores, two myosin-dependent activities. The chimeras' actin-activated ATPase rates, rates of ATP and ADP binding to S1, and rate of ATP-induced actin-S1 dissociation were identical to wild type's rates, indicating that the purified chimeras are functional and properly folded. However, the motility of the *Acanthamoeba* chimera was one-third of wild type's and the skeletal chimera's motility was equal to wild type's. The rate of ADP dissociation from S1 (k_4 of Scheme I) differed between the three, with wild type displaying an intermediate rate, and correlated with the extrapolated ADP off-rate from the ADP binding plot. While not a step in the actin-myosin cycle, it does indicate that Loop 1 changes in these chimeras affect interactions specifically with ADP.

Strikingly, the rate of ADP release from the actin-skeletal chimera complex (k_8) was twice as fast as from the actin-wild type complex, while the *Acanthamoeba* chimera's rate of ADP release from the actin-S1 complex was one-third of wild type's. This correlated well with the observed motility of the *Acanthamoeba* chimera, but not with the observed motility of the skeletal chimera. While there were no differences from wild type in the rate of actin-S1 dissociation (k_{10} of Scheme I), surprisingly, it is slow enough to be at least partially rate-limiting for velocity. It seems that for *Dictyostelium* myosin, Loop 1 does affect ADP release both in the presence and absence of actin, and this change can affect motility if subsequent steps are not significantly slower. Thus, the *Acanthamoeba* chimera's motility decreased as a result of the slowed ADP release rate, but an increase in motility of the skeletal myosin was not possible because the subsequent actin-myosin dissociation step is too slow. This study showed that Loop 1 is capable of both increasing and decreasing the ADP release rate and can influence motility, without changing other myosin activities. However, because the faster loop is longer and the slower loop is shorter than the *Dictysostelium* loop, the issue of the

effect of Loop 1 length vs. sequence on myosin activity was not resolved in this study. Additionally, the discovery that *Dictyostelium* myosin motility is not clearly limited by a single kinetic step makes the interpretation of motility results less straightforward. Comparison of data between labs regarding Loop 1 is made difficult by the fact that the activity of the loops may vary with the type of myosin used. Ideally, the *Dictyostelium* study would be extended to include long loops from slow myosins (e.g., the inserted and non-inserted smooth muscle loops) as well as length and charge changes to undertake a more systematic study of the loops' role. Additionally, structural and biophysical data regarding the flexibility of the loop and the nucleotide pocket would help explain the loop's function. The Loop 1 chimera studies support the hypothesis that this loop can affect ADP release and therefore can affect motility.

Crystal structure information

While proteolysis and crosslinking studies have provided structure-function information, until recently there has been little structural data available on the loops from crystallographic studies, presumably because of the loops' flexibility. Little resolution is available for Loop 2, and a co-crystal structure of the motor domain bound to actin, although difficult, would be ideal for visualizing these interactions. The recent publication of the crystal structure of the smooth muscle myosin motor domain and a complex with the essential light chain bound highlighted the possible role of Loop 1 in interactions with the essential light chain (Dominguez *et al*., 1998). Parts of Loop 1 are resolved in the MDE (*m*otor *d*omain with *e*ssential light chain) structure and the loop appears to be in contact with the "G helix" of the ELC, which has four glutamic acids that could interact with three positively-charged lysines in Loop 1. The authors suggest that the essential light chain may interact with Loop 1 at the beginning of the power stroke, which is the state they believe the smooth muscle myosin motor domain with MgADP · AlF_4^- and MgADP · BeF_x structures represent. Swinging of the ELC-bound lever arm is then limited by the presence of the *N*-terminal domain at the end of its stroke. This model implies that for some myosins, Loop 1 may play a role in the pre-stroke state, while much of the available kinetic data suggests that Loop 1 is important for the post-stroke regulation of ADP release. Both may be true for different myosins, and could be a reason for isoform diversity at Loop 1 that is separate from its effect on the rate of ADP release.

Conclusion

We have presented numerous studies of the two surface loops in the myosin head in order to make several points about their functions. Most importantly, the loops do appear to have specific activities and are important to the proper motor function of myosins. While at first glance, the loops appear to be nonconserved, they are in fact well conserved when grouped according to kinetic activities (Goodson *et al*., 1999). In addition to changing kinetic activities, they affect regulation (Rovner *et al*., 1995) and may provide additional binding sites for other proteins, including actin (Reinhard *et al*., 1995; Yazu *et al*., 1999). Sequence of the loops, rather than length, appears to be extremely important for optimal motor activity (Furch *et al*., 1998; Goodson *et al*., 1999; Murphy and Spudich, 1998, 1999; Sweeney *et al*., 1998). The Loop 1 and Loop 2 chimera studies in general support the ideas that (1) the nature of each loop affects specific steps of the actin-myosin ATPase cycle, (2) the loops may have slightly varying roles depending on the host myosin, and (3) Loop 2 generally influences the actin-activated ATPase activity through changes in both actin affinity and V_{max} while Loop 1 affects actomyosin's affinity for ADP.

In general, changing the loops provides a means of altering myosin's activities without changing the core functions of the molecule. Recently, a single-headed motor, KIF1A, was found to be processive, and it was postulated that a lysine-rich loop characteristic of monomeric KIFs acts as a microtubule anchor, allowing the protein to move to the next binding site without detaching (Okada and Hirokawa, 1999). Perhaps one of the myosins with an actin-binding insert could function in a similar way to move processively. Thus, changes at loops may be a general mechanism for motors and other enzymes to modify their activities without altering their core functions. Because it has been well characterized on many levels, myosin serves as an excellent model for elucidating the function of such variable surface loops.

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