Kinetic Tuning of Myosin via a Flexible Loop Adjacent to the Nucleotide Binding Pocket

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H. Lee Sweeney‡‡, Steven S. Rosenfeld‡, Fred Brown‡, Lynn Faust‡, Joe Smith‡, Jun Xing‡, Leonard A. Stein** and James R. Sellers‡‡

From the ‡Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, the Departments of *Neurology and Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, Alabama 35294, the **Department of Medicine, State University of New York, Stony Brook, New York 11794, and the ‡‡Laboratory of Molecular Cardiology, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

A surface loop (25/50-kDa loop) near the nucleotide pocket of myosin has been proposed to be an important element in determining the rate of ADP release from myosin, and as a consequence, the rate of actin-myosin filament sliding (Spudich, J. A. (1991) Nature 372, 515–518). To test this hypothesis, loops derived from different myosin II isoforms that display a range of actin filament sliding velocities were inserted into a smooth muscle myosin backbone. Chimeric myosins were produced by baculovirus/Sf9 cell expression. Although the nature of this loop affected the rate of ADP release (up to 9-fold), in vitro motility (2.5-fold), and the Vmax of actin-activated ATPase activity (up to 2-fold), the properties of each chimera did not correlate with the relative speed of the myosin from which the loop was derived. Rather, the rate of ADP release was a function of loop size/ flexibility with the larger loops giving faster rates of ADP release. The rate of actin filament translocation was altered by the rate of ADP release, but was not solely determined by it. Through a combination of solute quenching and transient fluorescence measurements, it is concluded that, as the loop gets smaller, access to the nucleotide pocket is more restricted, ATP binding becomes less favored, and ADP binding becomes more favored. In addition, the rate of ATP hydrolysis is slowed.

Resolution of the atomic structure of the myosin II motor from crystallographic x-ray diffraction data has created interest in several flexible loops on its surface. These include the "HCM" loop (1, 2) and two loops that were not resolved in the crystal structures and are the sites of proteolytic cleavage: the loops at the 25/50-kDa junction and 50/20-kDa junction. These junctional loops also have been referred to as loop 1 (25/50-kDa loop) and loop 2 (50/20-kDa loop). The HCM loop is so called because of a number of mutations at its base are associated with the human disease, hypertrophic cardiomyopathy (3). Furthermore, some members of the myosin family are regulated by phosphorylation of this loop (4, 5). Loops 1 and 2 have been proposed to be major determinants of the kinetic properties of myosin (6). Both loops are highly variable in sequence and length among members of the myosin II family. If this variability underlies a large degree of the kinetic diversity among myosins, then it would reveal an evolutionary strategy for kinetic tuning that would involve regions of the molecule outside of the core that might not have interactions with the backbone of the myosin motor. This could allow a rapid evolutionary divergence of motor properties without altering the core structure or the motor’s basic function.

The suggestion of a fundamental role for the junctional loops of myosin was based on a study of loop 2, which is located at the actin interface (7) and on the demonstration of functional differences for naturally occurring isoforms involving alternations in loop 1 (25/50-kDa loop) (8). The former study involved the creation of chimeric myosin II molecules that were the sequence of Dictostelium myosin II in all regions other than that of loop 2. The loop 2 sequences were derived from a number of other myosins. What was observed was an effect on the steady state ATPase activity. It was shown, at an actin concentration (20 μM) assumed to be high enough to reveal the Vmax of the actin-activated ATPase, that Vmax was correlated with that of the parent myosin from which the loop 2 sequence was derived. However, a subsequent study that performed similar experiments in the smooth muscle myosin II backbone concluded that loop 2 altered the apparent K0.5 for actin (KATPase), but not the Vmax of the steady state ATPase (9). Whether loop 2 affects only KATPase or Vmax or both, it clearly does alter the steady state ATPase activity of myosin. However, more extensive studies must be performed to determine whether or not it is the primary determinant of either the Vmax or K0.5.

The other junctional loop, loop 1 (25/50-kDa loop), is located near the catalytic site. Its sequence varies in vertebrate smooth and non-muscle myosin II isoforms as the result of alternative splicing, and these splice variants have been shown to lead to alterations in the steady state ATPase activity and rate of actin filament movement in an in vitro motility assay (8, 10). This loop has been proposed to be the primary determinant of the rate of ADP release from myosin, and as a consequence of this, the determinant of the in vitro actin filament sliding rate (6).

To examine the role of loop 1, we adopted the same general strategy that was previously used in evaluating loop 2 (7, 9). We took a common myosin II backbone (chicken smooth muscle myosin II), and inserted into it a number of 25/50-kDa loops that were based on the sequences found in other myosins. We expressed both S1 and HMM-like fragments of myosin, and

* The abbreviations used are: S1, myosin subfragment 1; HMM, heavy meromyosin; mant, methylanthraniloyl; DTT, dithiothreitol; MOPS, 4-morpholinopropanesulfonic acid; ELC, essential light chain; RLC, regulatory light chain.

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§ To whom correspondence should be addressed: Dept. of Physiology, University of Pennsylvania School of Medicine, 3700 Hamilton Walk, Philadelphia, PA 19104-6085. Tel.: 215-898-0485; Fax: 215-898-0475; E-mail: lsweeney@mail.med.upenn.edu.

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assessed their function, using steady state ATPase assays, stopped flow measurements of the rate of ADP release from myosin bound to actin, and the rate of actin filament sliding in an in vitro motility assay. Our findings suggest that this loop can alter all of these kinetic properties to a degree that suggests that it has a role in determining isoform diversity. However, it does not appear to be the major determinant of any of the kinetic properties of myosin. Thus, the 25/50-kDa loop appears to be a modulator of kinetic properties, which are primarily determined by other structural elements within the myosin motor. Insights into the mechanism of this kinetic modulation were obtained via a combination of solute quenching and nucleotide binding kinetics. The measurements suggest a model in which the relative mobility of the "switch" elements of the nucleotide binding pocket are modulated by the nature of the residues occupying the 25/50-kDa loop.

EXPERIMENTAL PROCEDURES

Construction of Chimeras—The cDNA for chicken gizzard smooth muscle myosin was truncated at either the codon corresponding to amino acid 559 to create a S1-like fragment, or at amino acid 1112, to create a recombinant fragment. In the case of the S1, a Flag peptide sequence (11), followed by a stop codon was appended, whereas for the HMM, a Myc epitope sequence (12), followed by the Flag sequence and a stop was appended. The Flag peptide allowed for affinity purification, whereas the Myc epitope was for motility studies. Both the S1- and HMM-like constructs were subcloned into the baculovirus transfer vector, pVL 1393 (Invitrogen). Two silent mutations were introduced into the S1 sequence to create unique restriction sites flanking the 25/50-kDa loop. Alternative loops were then introduced by using these sites to open the plasmid; a linker corresponding to the new loop flanked by the silent restriction sites was then ligated in place, creating the chimeric cDNA. The S1 chimeras was then used to generate the corresponding HMM. Generation of the recombinant baculoviruses coding for the S1- and HMM-like constructs followed published techniques (13).

Expression and Purification of Proteins—Baculovirus expression was used to produce S1- and HMM-like fragments of chicken smooth muscle myosin II. This involved infection of an insect cell line (Sf9) with recombinant baculovirus driving high level expression of foreign protein under the polyhedron promoter. Sf9 cells were co-infected with recombinant virus containing chicken smooth muscle (LC17b) and RLC cDNAs and with recombinant virus coding for a truncated myosin heavy chain. For a subset of experiments, virus coding for the alternative ELC (LC17a) was substituted.

Three days after infection, Sf9 cells were lysed in a high ionic strength buffer containing ATP, DTT, Nonidet P-40, and protease inhibitors. This was followed by low speed centrifugation, ammonium sulfate fractionation, and overnight dialysis at 4 °C in the presence of 0.1 mM EGTA, 1 mM DTT, 20 mM KCl, 20 mM Hepes, pH 7.4, and 1 mM MgCl2 at 25 °C. The equipment used for the myosin-mediated translocation of actin filaments and the method used for quantification of the motion have been described previously (22).

ATPase Assays of Phosphorylated HMM-like Fragments—The ATPase assays involved the measurement of the rate of radioactive ATP hydrolysis from γ-phosphate-labeled ATP, by the method of Pollard and Korn (23). The conditions of the assay were 2 mM MgCl2, 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM ATP at 25 °C. Vmax and KATPase were read from Lineweaver-Burk plots. The protein concentration used was over the range of 0–150 μM.

Solute Quenching—Solute accessibility of mann-ADP bound to S1 constructs was measured using acrylamide, as described previously (24).

Fluorescence Methodologies—Fluorescence lifetimes and anisotropy decays of mann nucleotide-labeled myosin constructs were performed as described previously (24). Anisotropy decay of mann-ADP was measured at 20.0 °C on a PRA 3000 single photon-counting system equipped with a DCM dye laser, the output of which was frequency-doubled to 325 nm as the excitation source for mann-ADP. The general procedures used to measure anisotropy decay and analyze the decay data have been described (25). The decay data from mann nucleotide were analyzed by a bi-exponential model.

RESULTS

Based on the structure of chicken skeletal muscle myosin S1 fragment (1) and on the structure of the Dictyostelium myosin motor domain (26), the boundaries of the 25/50-kDa loop were defined as illustrated by the underlined sequences in Fig. 1. The chimeric loops, shown below the two chicken smooth muscle wild type loops, are grouped according to relative myosin speed. The chicken fast skeletal, rat cardiac, and Dictyostelium myosins are all faster than smooth muscle myosin, whereas the
Muscle myosins gave ADP release and motility values for these loops were intermediate to the gizzard (phosphorylated) and the corresponding rate of release from smooth muscle myosin HMM-like constructs with the phosphorylated HMM. Fig. 2 depicts the rate of ADP activity in the absence of RLC phosphorylation. Constructs displayed movement or actin-activated ATPase activity in the presence of phosphorylation of the RLC. None of the HMM-like constructs shown displayed the rate of ADP release or ADP release from actin-myosin-ADP using expressed HMM (phosphorylated) fragments of chicken smooth muscle myosin incorporating the 25/50-kDa loop substitutions. The first two sequences are those of the two chicken smooth muscle wild type loops. These are followed by four loops from myosins that are faster than either of the smooth muscle wild types. These are followed by three loops from non-muscle myosins that are considerably slower than smooth muscle myosin. The last loop (D25/50) is a large deletion of the smooth muscle loop.

Three non-muscle myosins are all slower. All myosin S1 and HMM-like constructs containing either wild type or chimeric loops (Fig. 1) yielded 1–2 mg of active myosin from 1 liter of Sf9 cells (approximately $3 \times 10^6$ cells). For all S1 and HMM-like constructs, the purified myosin fragments displayed a heavy chain:ELC:RLC stoichiometry of 1:1:1, as measured by densitometry (correcting for molecular weight differences; data not shown). All values given for HMM-like constructs are in the presence of phosphorylation of the RLC. None of the HMM-like constructs displayed movement or actin-activated ATPase activity in the absence of RLC phosphorylation.

Table I presents the values for the rate of ADP release from either smooth muscle myosin S1 or phosphorylated HMM bound to actin. The values were not different for S1 compared with the phosphorylated HMM. Fig. 2 depicts the rate of ADP release from smooth muscle myosin HMM-like constructs (phosphorylated) and the corresponding rate of in vitro translocation of actin filaments by each of the constructs. The fastest values for both parameters were obtained with the gizzard loop. Loops from the faster myosins gave a range of ADP release values that were similar to or slower than that obtained for the chicken aorta smooth muscle myosin loop. The in vitro motility values for these loops were intermediate to the gizzard and aorta values. Interestingly, the loops from the slower non-muscle myosins gave ADP release and in vitro motility values that were as fast or faster than the those obtained with the aorta loop. To facilitate comparison of the in vitro motility measurements of the chimeric myosins (Fig. 2), literature values for the parent myosins are given in Table II. The comparison reveals that there is no correlation between the speed of the chimeric myosins and the speed of the myosin from which the 25/50-kDa loops were derived ("loop donors").

The only obvious correlation between the loop sequences and any of the measured kinetic parameters was a correlation between the length of the 25/50-kDa loop and the rate of ADP release. This relationship is depicted in Fig. 3A. Note that the only loop from Fig. 1 that does not fit this size rule is the gizzard wild type loop.

This was further examined by constructing two additional smooth muscle myosin chimeras for which the 25/50-kDa loops are shown in Fig. 3B. The first substituted the seven amino acid alternative smooth muscle insertion from rabbit in place of that of chicken. The second replaced five of the seven gizzard insert residues with alanines. As shown in Fig. 3A, the alanine replacement decreased the rate of ADP release to a value that better fits the size rule whereas the rabbit loop gave a value lower than the chicken gizzard value, but higher than the loop containing the five alanine substitutions. The corresponding motility and ATPase data for HMM-like fragments containing the same altered inserts of Fig. 3 are given in Table IV.

Table III presents the results of actin-activated ATPase as-
**Table II**

| Protein Type                        | μm/s |
|-------------------------------------|------|
| Chicken gizzard wild type           | 1.15 |
| Chicken aorta wild type             | 0.60 |
| Chicken fast skeletal               | 9.0  |
| Rat α-cardiac                       | 4.8  |
| Rat β-cardiac                       | 2.4  |
| Dictyostelium                       | 2.2  |
| Human non-muscle A                  | 0.3  |
| Chicken non-muscle B                | 0.1  |
| Xenopus non-muscle B                | 0.22 |

*5 mM KCl.

**Table III**

| Myosin II source of 25/50-kDa loop | ATPase V\text{max} | ATPase K\text{m} |
|------------------------------------|---------------------|-----------------|
| Chicken gizzard smooth muscle      | 1.24 ± 0.10         | 57 ± 15         |
| Chicken aorta smooth muscle        | 0.58 ± 0.11         | 56 ± 19         |
| Chicken fast skeletal              | 0.81 ± 0.04         | 19 ± 8          |
| Rat cardiac α                      | 0.50 ± 0.05         | 64 ± 11         |
| Rat cardiac β                      | 0.53 ± 0.13         | 50 ± 18         |
| Dictyostelium                      | 0.87 ± 0.08         | 43 ± 12         |
| Chicken non-muscle A               | 0.84 ± 0.18         | 85 ± 19         |
| Chicken non-muscle B1              | 0.86 ± 0.16         | 108 ± 8         |
| Xenopus non-muscle B1              | 0.83 ± 0.10         | 59 ± 12         |
| Δ25/50                             | 0.86 ± 0.09         | 19 ± 10         |

Although the K\text{m} values showed considerable variability, the chicken fast skeletal loop and the deletion (Δ25/50) loop yielded significantly lower K\text{m} values than either wild type or for any of the other chimeras.

To address whether the correspondence between size of the loop and the rate of ADP release was related to loop flexibility and size, two additional smooth muscle myosin HMM chimeras were constructed. Into the gizzard 25/50-kDa loop, three proline substitutions were made, and into the deletion (Δ25/50) loop, one proline substitution was made. These are shown in Fig. 4. The effect of the proline insertion was, in both cases, to reduce the speed of ADP dissociation from the actin-myosin HMM-ADP complex. For the gizzard loop, the reduction was from 79 ± 5 s\(^{-1}\) to 37 ± 5 s\(^{-1}\). For the deletion loop, the reduction was from 12 ± 2 s\(^{-1}\) to 8 ± 2 s\(^{-1}\).

It was noted that all of the loops from myosin II isoforms had positively charged residues near the N-terminal end of the loop, and all of the loops had a net positive charge. To examine the possible contribution of this charge, the three lysines of the gizzard loop were replaced with either aspartate or alanine residues, as depicted in Fig. 5. Either removal or reversal of this charge resulted in a decrease in both the V\text{max} and the K\text{m} of the actin-activated ATPase activity (Table IV). Charge removal had no effect on the rate of ADP release, whereas charge reversal slowed the release (Table IV). The in vitro motility decreased in both cases, but to a much greater extent in the case of charge reversal (Table IV).

Two ELC isoforms are associated with smooth muscle myosin, which previous studies have examined for functional significance (8, 10). We extended these previous studies by substituting LC\text{17b} for LC\text{17a} in preparations of HMM with the gizzard, aorta, and deletion loops. The values for the rate of ADP release, in vitro motility, and actin-activated ATPase activity are given in Table V.

To provide further insights into the mechanism underlying kinetic modulation via the 25/50-kDa loop, the kinetics of binding of ATP and 2′-deoxy-mant-ADP to S1(gizzard loop), S1(aorta loop), and S1(loop deletion; Δ25/50) were measured in the stopped flow. Binding of ATP was monitored at 10 °C by the increase in tryptophan fluorescence emission (32). A plot of apparent rate versus [ATP] yielded data that could be fit to a rectangular hyperbola (Fig. 6) for the constructs, defining maximum rates (V\text{max}) and apparent dissociation constants (K\text{d}) (Table VI). The maximum rates for the S1(gizzard) construct was 32% larger than that for the S1(Δ25/50) construct, and 95% larger than the S1(aorta) construct. This maximum rate of tryptophan fluorescence enhancement is thought to represent the ATP hydrolysis rate (32.) Furthermore, the apparent dissociation constant was approximately 5 times larger for S1(Δ25/50), whereas both the gizzard and aorta constructs had similar values for their dissociation constants. This difference was also seen for HMM(gizzard loop) and HMM(Δ25/50) at 20 °C using the fluorescent ATP analogue, mant-ATP (Fig. 7). Binding of
mant-ADP. Kinetics of binding of this ligand were monitored by a single exponential term. Although the values of mant-ATP produced a fluorescent enhancement that could be fitted to a single exponential process, and the apparent rate constant was plotted versus final [ATP]. Data was fitted to a rectangular hyperbola, defining apparent binding constants and maximum rates summarized in Table VI.

**TABLE VI**

| Alterations in the 25/50-kDa loop | Rate of ADP release | In vitro motility | ATPase $V_{\text{max}}$ | ATPase $K_m$ |
|-----------------------------------|---------------------|-------------------|------------------------|-------------|
|                                   | $s^{-1}$            | $\mu$m/s          | $s^{-1}$               | $\mu$m      |
| Chicken gizzard wild type         | 79 ± 5              | 1.15 ± 0.19       | 1.24 ± 0.10            | 57 ± 15     |
| Chicken aorta wild type           | 24 ± 3              | 0.61 ± 0.13       | 0.58 ± 0.11            | 56 ± 19     |
| Rabbit smooth insert (Fig. 3)     | 44 ± 3              | 0.84 ± 0.17       | 0.76 ± 0.08            | 67 ± 13     |
| Alanine substitution (Fig. 3)     | 34 ± 3              | 0.59 ± 0.05       | 0.43 ± 0.06            | 13 ± 5      |
| Lysines to aspartates (Fig. 5)    | 22 ± 5              | 0.42 ± 0.14       | 0.81 ± 0.04            | 19 ± 8      |
| Lysines to alanines (Fig. 5)      | 77 ± 6              | 0.63 ± 0.14       | 0.80 ± 0.05            | 64 ± 11     |

**FIG. 6.** The kinetics of enhancement of tryptophan fluorescence produced by binding of ATP to gizzard S1 (open squares), Δ25/50 deletion mutant on a gizzard backbone (closed squares), and 25/50 aorta loop on a gizzard backbone (open triangles), 10 °C. A solution of S1 in 25 mM Hepes, 20 mM KCl, 1 mM MgCl₂, 1 mM DTT, pH 7.5, was mixed with an equal volume of an at least 20-fold molar excess of ATP in the same solution. The rate of the resulting tryptophan fluorescence enhancement was fitted to a single exponential process, and the apparent rate constant was plotted versus final [ATP]. Data was fitted to a rectangular hyperbola, defining apparent binding constants and maximum rates summarized in Table VI.

**TABLE V**

| Alterations in the 25/50-kDa loop and ELC isoform | Rate of ADP release | In vitro motility | ATPase $V_{\text{max}}$ | ATPase $K_m$ |
|-----------------------------------------------|---------------------|-------------------|------------------------|-------------|
|                                               | $s^{-1}$            | $\mu$m/s          | $s^{-1}$               | $\mu$m      |
| Chicken gizzard wild type + LC₁₇a             | 79 ± 5              | 1.15 ± 0.19       | 1.24 ± 0.10            | 57 ± 15     |
| Chicken aorta wild type + LC₁₇a               | 24 ± 3              | 0.61 ± 0.13       | 0.58 ± 0.11            | 56 ± 19     |
| Δ25/50 + LC₁₇a                                | 12 ± 2              | 0.56 ± 0.18       | 0.86 ± 0.09            | 19 ± 10     |
| Chicken gizzard wild type + LC₁₇b             | 77 ± 4              | 1.18 ± 0.21       | 1.20 ± 0.15            | 59 ± 17     |
| Chicken aorta wild type + LC₁₇b               | 23 ± 3              | 0.59 ± 0.19       | 0.61 ± 0.13            | 55 ± 24     |
| Δ25/50 + LC₁₇b                                | 13 ± 3              | 0.54 ± 0.20       | 0.84 ± 0.11            | 22 ± 9      |

**FIG. 7.** The kinetics of enhancement of mant-ATP fluorescence emission produced by mant-ATP binding to the catalytic site gizzard HMM (open squares) and the Δ25/50 deletion mutant on a gizzard HMM backbone (open circles), 20 °C. A solution of HMM or deletion mutant in 25 mM Hepes, 20 mM KCl, 1 mM MgCl₂, 1 mM DTT, pH 7.5, was mixed with an equal volume of an at least 20-fold molar excess of mantATP in the same solution. The mant fluorophor was excited at 295 nm through energy transfer from a myosin tryptophan residue. The rate of the resulting fluorescence enhancement was fitted to a single exponential process, and the apparent rate constant was plotted versus final [mantATP]. Data was fitted to a rectangular hyperbola, defining apparent binding constants and maximum rates summarized in Table VI.

mant-ATP produced a fluorescent enhancement that could be described by a single exponential term. Although the values of $\lambda_{\text{max}}$ were very similar, the value of $K_d$ for HMM(Δ25/50) was over 2-fold larger.

By contrast, the situation appears reversed for 2'-deoxy-mant-ADP. Kinetics of binding of this ligand were monitored by energy transfer from protein tryptophan to the mant fluorophor. A maximum rate was not observed over the concentration range examined (Fig. 8). The slopes of rate versus nucleotide concentration define apparent second order rate constants ($k_a$), back extrapolation of the rate defines apparent dissociation rates ($k_d$), and the ratio of $k_a/k_d$ defines apparent dissociation constants ($K_d$) (Table VII). This reveals that compared with S1(gizzard), the S1Δ25/50 construct binds 2'-deoxy-mant-ADP with a 5-fold higher $k_a$, with a 13-fold lower $k_d$, and with a 70-fold lower $K_d$.

These results suggest that deletion of the 25/50 loop shifts the equilibrium of S1 conformations toward one that favors ADP binding, and should consequently favor a "strong binding" state. To explicitly test this, S1(gizzard)/2'-deoxy-mant-ADP and S1D25/50/2'-deoxy-mant-ADP were mixed in the stopped
flow with at least a 15-fold molar excess of actin + 1 mM ATP. A plot of rate versus [actin] (Fig. 9) could be fit to a rectangular hyperbola for S1Δ25/50, defining values of $k_{\text{on}}$ for nucleotide release and $K_d$ for binding to actin. A maximum rate could not be observed for S1(gizzard), and a plot of rate versus [actin] was linear. A comparison of the initial slope of the hyperbolic plot for S1Δ25/50 with that for S1(gizzard), however, reveals that the apparent second order rate constants are very similar to each other (Fig. 9). In this experiment, the rates of release of 2'-deoxy-mant-ADP from S1(gizzard) and S1Δ25/50 were measured directly by mixing S1(gizzard)2'-deoxy-mant-ADP and S1Δ25/502'-deoxy-mant-ADP with 1 mM ATP. The release transients fit single exponential decays, with rate constants of 7.8 s$^{-1}$ and 21.1 s$^{-1}$ for S1(gizzard) and S1Δ25/50, respectively, which are in excellent agreement with values calculated from Fig. 8.

Additional information about the effect of the loop on the geometry of the catalytic site can be obtained by examining the spectroscopic properties of S1 constructs labeled in the catalytic site with fluorescent nucleotide, such as mant-ADP. In aqueous solution, the fluorescence decay of free mant-ADP can be described by a single lifetime of 4.0 ns (33). By contrast, the fluorescence decay of complexes of mant-ADP with either S1(gizzard) or S1Δ25/50 could be described by two lifetimes. For S1(gizzard), the lifetimes had values of 10.2 ± 0.2 ns and 1.8 ± 0.1 ns and with relative contents of 0.56 and 0.44, respectively. For S1Δ25/50, the corresponding values were 7.7 ± 0.2 ns and 2.1 ± 0.1 ns and with relative contents of 0.70 and 0.30, respectively. Addition of beryllium sulfate to 0.2 mM and sodium fluoride to 5 mM produces a myosin-ADP-BeF complex that mimics the pre-hydrolytic state (26). Lifetimes of mant-ADP bound to S1(gizzard) or S1Δ25/50 in the presence of beryllium fluoride were very similar to each other: 9.0 ± 0.1 ns and 3.8 ± 0.1 ns for S1(gizzard) with relative contents of 0.75 and 0.25, and 9.0 ± 0.1 ns and 3.5 ± 0.1 ns for S1Δ25/50 with relative contents of 0.78 and 0.22. mant-ADP consists of a mixture of the 2' and 3'-isomers (34). To avoid the confounding effects of a stereoisomeric mixture of fluorophors, fluorescence lifetimes were measured for S1(gizzard) or S1Δ25/50 complexed to 2'-deoxy-mant-ADP. This revealed that the fluorescence decays of both S1(gizzard)2'-deoxy-mant-ADP and S1Δ25/502'-deoxy-mant-ADP could still be characterized by two components. For S1(gizzard), the lifetimes were 8.73 ± 0.2 ns (70%) and 1.8 ± 0.1 ns (30%), whereas for S1Δ25/50 they were 8.2 ± 0.5 ns (30%) and 3.8 ± 0.3 ns (70%).

As a fluorescence quencher, acrylamide shortens the fluorescence lifetimes of bound mant-ADP as described by the Stern-Volmer equation (35), where $\tau_0$ is the singlet lifetime in the absence of quencher, $\tau$ is the lifetime at quencher concentration [Q], and $k_q$ is the bimolecular Stern-Volmer quenching rate constant. The quenching rate constant $k_q$ can vary from the rate of diffusion (totally accessible to solute) to zero (inaccessible). Thus, the effect of acrylamide on fluorescence lifetime can be used to measure the effect of the 25/50 loop deletion on the accessibility of bound nucleotide to solvent. Quenching studies were performed on S1(gizzard)mant-ADP, S1Δ25/50mant-ADP, S1-mant-ADP-BeF, and S1Δ25/50-mant-ADP-BeF. Results are summarized in Table IX. As has been reported previously for smooth muscle myosin (24), beryllium fluoride

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**Figure 8.** The kinetics of enhancement of 2'-deoxy-mant-ADP fluorescence emission produced by mant-ADP binding to the catalytic site gizzard S1 (open squares) and the Δ25/50 deletion mutant on a gizzard S1 backbone (open circles), 20 °C. Conditions are as detailed in Fig. 7. The rate of 2'-deoxy-mant-ADP fluorescence enhancement was found to have a linear dependence on nucleotide concentration. The slope of these curves defines apparent second order rate constants, $k_a$, and extrapolation to zero ligand concentration defines apparent dissociation rate constants, $k_d$, which are summarized in Table VII.

**Figure 9.** Dependence of the rate of 2'-deoxy-mant-ADP release from gizzard S1 (open squares) and the Δ25/50 deletion mutant on a gizzard S1 backbone (open circles) on actin concentration. A solution of 0.5–5 μM S1 + 25 μM 2'-deoxy-mant-ADP was mixed in the stopped flow with a 10-fold molar excess of actin + 1 mM ATP. The mant fluorescence emission was monitored by energy transfer from an S1 tryptophan residue, and the resulting fluorescence intensity decrease was fitted to a single exponential process. Data were fitted to a rectangular hyperbola, defining apparent binding constants, and maximum rates summarized in Table VIII. Conditions are as follows: 25 mM Hepes, 20 mM KCl, 1 mM MgCl$_2$, 1 mM DTT, pH 7.5, 20 °C.

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**Table VII**

| Reaction | $k_a$ (s$^{-1}$) | $k_d$ (s$^{-1}$) | $K_d$ (μM) |
|----------|----------------|----------------|-----------|
| S1(gizzard) + 2'-deoxy-mant-ADP | $3.3 \times 10^4$ | $6.2 \times 10^7$ | 188 |
| S1Δ25/50 + 2'-deoxy-mant-ADP | $1.7 \times 10^5$ | $5.0 \times 10^5$ | 2.8 |

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**Table VIII**

| Reaction | $\lambda_{\text{max}}$ (s$^{-1}$) | $K_d$ (μM) |
|----------|----------------|-----------|
| S1 + 2'-deoxy-mant-ADP × actin | 66.4 | 110 |
| S1Δ25/50 + 2'-deoxy-mant-ADP × actin | 5.6 | 12.6 |
reduces the solvent accessibility of the mant fluorophor when bound to S1 (gizzard) by approximately a factor of 2, whereas it has much less of an effect on S125/50-mantADP. Furthermore, the values of \( k_q \) for S1 (gizzard)-mant-ADP-BeF are very similar to those for S125/50-mantADP (Table IX).

Rotational correlation times were calculated from the anisotropy decays of S1 (gizzard)-mant-ADP-BeF and S125/50-mant-ADP-BeF. Both samples had similar limiting anisotropies (0.21 for S125/50-mant-ADP-BeF and 0.23 for S1-mant-ADP-BeF). The anisotropy decay for both samples was characterized by a single rotational correlation time that was 10% smaller for S125/50 construct (144.9 ± 4.0 ns for S1 versus 130.1 ± 3.6 ns for S125/50).

**DISCUSSION**

The data shown in Fig. 2 and Tables I and III demonstrate that the sequence of the 25/50-kDa loop alters the rate of ADP release from myosin bound to actin over a 9-fold range; the rate of *in vitro* motility, as measured by actin sliding velocity, over a 2.7-fold range; and the steady state ATPase activity over a 2-fold range. The magnitude of the alterations are less than an order of magnitude, yet the *in vitro* motility rates for the myosins from which the chimeric loops were derived vary by 90-fold (Table II). This suggests that this loop plays a modulatory role in the generation of isoform diversity, and is not the primary determinant of any of these kinetic properties. Furthermore, none of the loops produced any kinetic changes that were faster than those associated with the gizzard loop, suggesting that other elements of the myosin molecule ultimately limit all of the kinetic parameters as compared with those of faster myosins. Additionally, the data summarized in Table V demonstrate that none of the kinetic properties measured were altered by the ELC isoform (LC17a versus LC17b). This confirms and extends published reports (8, 10).

It is noteworthy than none of the loop substitutions resulted in a loss of RLC phosphorylation-mediated regulation of the HMM-like constructs. This is despite the fact that several of the loop substitutions resulted in a marked decrease in the \( K_{ATPase} \). In the study of Rovner et al. (9), chimeric substitutions in the 50/20-kDa loop caused a decrease in the \( K_{ATPase} \), and a loss of regulation. Based on the present study, one must conclude that decreasing \( K_{ATPase} \) does not in itself cause a loss of regulation, and that the loss of regulation seen in that study must be specific to alterations of the 50/20-kDa loop.

If one focuses on the rate of ADP release and *in vitro* motility, it is evident that loops derived from myosin molecules that are faster than smooth muscle myosin all produced values that were intermediate to the smooth gizzard and aorta wild type myosins. Although there is no correlation between the speed of the chimeric molecule and the speed of the parent molecule from which the loop was derived, there is a striking correlation between the rate of ADP release and the length of the 25/50-kDa loop (Fig. 3).

Although the shortest loops were associated with the slowest rates of actin filament translocation, velocity was not strictly proportional to the rate of ADP release. For example, the deletion loop and *Dictyostelium* loop gave rise to *in vitro* motility values that were similar to the longer aorta loop, even though the corresponding values of ADP release rate were much less for the deletion and *Dictyostelium* loops. Both the deletion loop and *Dictyostelium* loop give rise to steady state ATPase \( V_{max} \) values that are greater than that for the longer aorta loop. One possible interpretation is that, whereas the rate of ADP release can limit the shortening \( V_{max} \) of a muscle (and presumably *in vitro* motility), as has been suggested previously (36), it is not the sole determinant. Earlier steps in the crossbridge cycle may also contribute to the rate of unloaded actin-based movement.

The one loop sequence that is much faster in terms of ADP release rate than would be predicted from its length is the chicken gizzard loop. The chimeric substitutions shown in Fig. 3 reveal that this deviation is due to specific amino acids within the seven-amino acid insertion that distinguishes this loop from that of the aorta smooth muscle myosin II loop sequence. When the seven-amino acid insert was altered to that found in rabbit smooth muscle, both the rate of ADP release and *in vitro* motility declined. When multiple alanine substitutions were made (Fig. 3), the loop obeyed the same size rule as all other loops. This may indicate that the kinetic properties are not a function of loop size, but rather of loop flexibility, and that the seven-amino acid insert in the gizzard sequence confers a greater flexibility to the loop than do the substitutions of Fig. 3. If that is the case, then insertions that decrease the flexibility of the loop should slow the ADP release rate. To test this, the substitution of prolines was performed with two different loops within HMM constructs. In both cases, slowing of ADP release accompanied the proline insertions. This result supports the concept that it is loop flexibility that is the important property governing the release rate of ADP from myosin bound to actin. The data in Table IV demonstrate that charge reversal, but not charge removal, also slowed the rate of ADP release. It is interesting to note that two possible 25/50-kDa loops can be inserted into the *Placopesten* myosin II heavy chain, creating either the striated muscle or the slower, catch muscle myosin (37). The catch loop is one amino acid shorter and substitutes two proline residues for two charged residues.

How can one envision a mechanism whereby a loop that is near the nucleotide binding pocket controls the rate of ADP release? One possibility is that the longer loops extend to the nucleotide pocket, where they interact directly with ADP in a manner that accelerates ADP release. However, this is not supported by data that examined access of the nucleotide pocket to solvent for the gizzard loop versus the loop deletion (Table IX). As the data in Table IX indicate, deletion of the 25/50 loop reduces solute accessibility of the mant fluorophor by a factor of 2–3, a reduction in accessibility similar to that produced by addition of beryllium fluoride. This suggests that deletion of the loop restricts the “opening” of the nucleotide pocket when ADP occupies the active site, increasing nucleotide affinity, and reducing the rate of ADP release, both in the absence and presence of actin (Fig. 9).

The dissociation constant for 2'-deoxy-mant-ADP for S1 calculated from the kinetic data, at 188 \( \mu \)M, is over 4-fold larger than that directly measured for acto-S1 (38). This implies that following initial binding of ligand, the S1-nucleotide complex undergoes a conformational change that is not detected by energy transfer-induced fluorescence from the mant nucleotide. This is consistent with the lifetime data, which does not detect substantial amounts of free nucleotide at total S1 and ligand concentrations of 50 \( \mu \)M, but which indicates that S12'-deoxy-mant-ADP is a mixture of two states with an equilibrium constant of approximately 4. Deletion of the 25/50 loop reduces
this equilibrium constant to approximately 0.4. However, ADP affinity is still enhanced, due both to acceleration of the forward rate constant and reduction in the reverse rate constant for the ligand binding step. Previous work by Marston and Taylor (39) measured a rate of ADP release of 0.9 s\(^{-1}\) for proteolytically prepared gizzard S1, which is approximately 9 times slower than what was measured in this study. This is consistent with our recent finding that nucleotide release from recombinant S1 is 8–10 times faster than that for proteolytic S1, perhaps because heavy chain of the former is uncleaved.\(^2\) By contrast, ATP binding and hydrolysis, as measured by the enhancement of tryptophan fluorescence (32, 40), is slowed for the deletion mutant and the ATP affinity is reduced by a factor of 5.

Results of transient fluorescence measurements suggest a basis for these effects on nucleotide binding. For S1(gizzard)\(\rightarrow\)deoxy-mant-ADP, the major component in the fluorescence decay was that with the longer lifetime (8.7 ns), whereas the situation was reversed for the deletion mutant. The conformation of S1-nucleotide is an equilibrium mixture of “strong” and “weak” states (41). Thus, the fluorescence decay results suggest that deletion of the 25/50 loop shifts the equilibrium to a strong state.

Deletion of the 25/50 loop had a marked effect on the kinetics of actin-activated mant-ADP release (Fig. 9). Data for both S1(gizzard)S1\(\Delta 25/50\) are consistent with the following model (Scheme 1), where the asterisk refers to states of enhanced fluorescence.

$$K_{\text{max}} \rightleftharpoons k_{\text{off}} k_{\text{on}} [\text{actin}] \rightleftharpoons A:M:A:D*$$

**REACTION 1**

In this scheme, \(K_{\text{on}}\) is a rapid equilibrium relative to the second step, the apparent second order rate constant, \(k_{\text{on}} = K_{\text{on}} k_{\text{on}}\), and the maximum rate for the fluorescence transient \(\lambda_{\text{max}} = k_{\text{2}}\), because under the conditions of the experiment, dissociation of actin-nucleotide is irreversible. A plot of rate \(k_{\text{on}}\) [actin] for S1(gizzard) is linear; hence, \(\lambda_{\text{max}}(\text{gizzard}) \gg \lambda_{\text{max}}(\Delta 25/50)\). However, \(k_{\text{off}}(\text{gizzard}) \approx k_{\text{off}}(\Delta 25/50)\), which implies that \(K_{\text{max}}(\text{gizzard}) < K_{\text{max}}(\Delta 25/50)\). Thus, deletion of the 25/50 loop has two effects; it enhances actin affinity for S1-ADP, as would be expected if this construct is largely in a strong binding state, and it markedly slows the rate of actin-accelerated nucleotide release. Taken together, these results suggest that deletion of the loop locks the nucleotide pocket into a state that strongly binds ADP, and restricts solute entry. However, this conformation of the pocket retards ATP entry into the active site and slows hydrolysis.

A structural basis for this type of mechanism is suggested by a close examination of the location of this loop. The 25/50-kDa loop connects two helices that are attached to key elements of the nucleotide binding pocket. At the N-terminal end of the first helix is the P-loop, and at the C-terminal end of the second helix begins a sequence that ultimately leads to the switch I component of the active site (42). Although there is currently not a structure available for myosin in the absence of both nucleotide and metal at the active site, there are such structures for a number of G proteins (43). What these structures suggest is that the release of MgADP may come about by breaking the coordination of the P-loop with the magnesium ion. This could be accomplished in myosin by a movement of the helix that contains amino acid T186 and K185 (Dictyostelium myosin II amino acid numbers). One can envision that as the flexibility of the 25/50-kDa loop increases, the probability that the helix will move via thermal motion sufficiently to release the MgADP is increased. Interestingly, the crystal structure of kinesin reveals that its P-loop helix is interrupted by a loop (44). Perhaps that loop in kinesin plays a functional role that is analogous to the 25/50-kDa loop of myosin.

In 1967, Barany (45) suggested that the same kinetic step that determines the \(V_{\text{max}}\) of the solution ATPase activity of myosin also determines the maximal velocity of shortening. This was based on correlations between the two parameters in myosins from a large number of different muscles. The data from this study demonstrate that the rate of \(V_{\text{max}}\) motility (the \(V_{\text{max}}\) analog of the shortening \(V_{\text{max}}\) of a muscle) and the ATPase \(V_{\text{max}}\) can be dissociated from each other. However, if one examines the data from the three smooth muscle wild type loops (chicken gizzard, chicken aorta, and rabbit; Table IV), the proportionality noted by Barany is maintained. Thus, although there is no fundamental mechanistic link between the two parameters, Barany’s observed correlation may reflect evolutionary pressure to maintain proportionality between these parameters. This may reflect interaction distance and duty cycle constraints on the myosin motor that must be maintained within a narrow window to achieve optimal muscle performance.

In summary, the nature of the loop at the 25/50-kDa junction alters the kinetic properties of myosin in a manner that allows for the generation of isoform diversity. The greatest effect of this loop is on the rate of ADP release, which seems to be determined by the flexibility of the loop; greater flexibility leads to an enhancement in the rate of ADP release. The loop can also alter the steady state ATPase activity (both \(V_{\text{max}}\) and the apparent \(K_m\)) and the rate of ATP hydrolysis. The speed of actin filament translocation is primarily determined by the rate of ADP release, with contributions from earlier steps. Thus, although this loop is not the primary determinant of shortening speed of myosin, as was hypothesized, it is an important modulatory element of myosin kinetics. It is likely that the kinetic properties of myosin will be the sum total of numerous such modulatory regions that must be altered in concert to achieve the functional range that the myosin motor manifests.

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