Effects of SH1 and SH2 Modifications on Myosin Similarities and Differences

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ABSTRACT The properties of myosin modified at the SH2 group (Cys-697) were studied and compared with the previously reported properties of myosin modified at the SH1 group (Cys-707). 4-[*N*-[(iodoacetoxy)ethyl]-*N* methylamino]-7-nitrobenz-2-oxa-1,3-diazole (IANBD) was used for selective modification of the SH2 group on myosin. SH2-labeled heavy meromyosin (SH2-HMM), similar to SH1-labeled HMM (SH1-HMM), did not propel actin filaments in the in vitro motility assays. SH1- and SH2-HMM produced similar amounts of load in the mixtures with unmodified HMM; the sliding speed of actin filaments gradually decreased with an increase in the fraction of either one of the modified HMMs in the mixture. In analogy to SH1-labeled myosin subfragment 1 (SH1-S1), SH2-labeled S1 (SH2-S1) activated regulated actin in the in vitro motility assays. SH2 modification inhibited Mg-ATPase of S1 and its activation by actin. The weak binding of S1 to actin was unaffected whereas the strong binding was weakened by SH2 modification. Overall, our results demonstrate similar behavior of SH1- and SH2-modified myosin heads in the in vitro motility assays despite some differences in their enzymatic properties. The effects of these modifications are ascribed to the location of the SH1-SH2 helix relative to other functional centers of S1.

INTRODUCTION

The SH1 (Cys-707) and SH2 (Cys-697) groups are the two most reactive cysteines on the myosin head (S1) and can be selectively labeled with thiol reagents. SH1 and SH2 groups are located on the opposite ends of a short α-helix in the catalytic domain of the myosin head and are separated from one another by ~19 Å (Rayment et al., 1993). This helix (see Fig. 1) is believed to play a key role in the conformational changes that occur in the myosin head during the force generation coupled to ATP hydrolysis. The first evidence of the mobility of this helix came from cross-linking experiments. It was shown that SH1 and SH2 groups can be cross-linked by reagents with widely varying cross-linking spans (5 Å to 12–14 Å), and even by disulfide bond formation, and that binding of nucleotides to S1 promotes such cross-linking. This helix appeared also to be functionally important; the ATPase activity of S1 was inactivated by its cross-linking (Reisler et al., 1974b; Burke and Reisler, 1977; Wells et al., 1980), and nucleotides, if present, became noncovalently trapped in the active site (Wells and Yount, 1980).

Other indications of functional and structural importance of this region came from studies on the selective modification of the SH1 group on S1. It was shown that this modification affects strongly the Mg-ATP hydrolysis cycle of S1, mostly by accelerating the release steps of ATP hydrolysis products (Sleep et al., 1981; Ostap et al., 1993). The activation of Mg-ATPase activity of S1 by actin was also altered greatly by SH1 modification. Mulhern and Eisenberg (1978) showed that such activation was almost abolished irrespective of the type of label attached to SH1. More recent studies showed that the motor function of myosin heads in the in vitro motility assays was blocked by SH1 modification (Root and Reisler, 1992; Marriott and Heidecker, 1996; Bobkov et al., 1997). Moreover, the work of Bobkov et al. (1997) revealed that SH1 modification enhanced the ability of S1 to activate regulated actin. The conformation of S1 alone and in complexes with nucleotides, as detected by differential scanning calorimetry study, was also altered by SH1 modification (Golitsina et al., 1996). According to these studies, the most pronounced effect of SH1 modification was on the conformation of S1 in the complex with ADP and V_i, i.e., in the analog state corresponding to the intermediate complex S1-ADP-P_i in the Mg-ATP hydrolysis cycle of S1.

The data described above show that the SH1-SH2 helix is a functionally important site on S1, but the mechanism by which this helix is involved in the force generation cycle is still unknown. It was suggested on the basis of three-dimensional structures of the chicken skeletal S1 (Rayment et al., 1993) and truncated *Dictyostelium* S1 (Fisher et al., 1995) that during the power stroke the light-chain-binding domain (LCBD) swings relative to the catalytic domain of S1, acting like a lever arm. Experiments with S1 constructs containing shortened or elongated LCBDs (Uyeda et al., 1996) and EM reconstruction of actin filaments decorated by S1 (Whittaker et al., 1995) led to the conclusion that the pivot point of this swinging motion is in the vicinity of the SH1-SH2 helix. If we assume that conformational changes in the SH1-SH2 helix are involved in lever arm movement, then the cross-linking of SH1 and SH2 groups or modifica-

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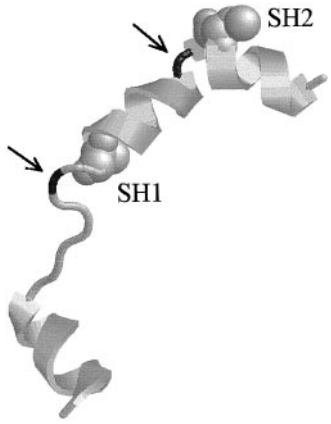

FIGURE 1 Representation of the SH1-SH2 helix based on the crystal structure of myosin subfragment 1 (Rayment et al., 1993). SH1 (Cys-707) and SH2 (Cys-697) groups are shown in a space-filled mode. The arrows mark positions of two conserved glycines, 699 (upper) and 710 (lower).

tion of SH1 can disrupt the mechanical function of S1 by altering the flexibility of this helix or its coupling to the lever arm.

Although the effects of SH1 modification on S1 properties were studied extensively, little is known about the effects of SH2 modification on S1. Such information is important, as a comparison of the effects of SH1 and SH2 modifications on the structure and function of S1 could provide new insights into the role of the SH1-SH2 helix in the generation of force. In this study, we examined the effects of SH2 modification on the motor, regulatory, and catalytic properties of S1 and compared them with the effects of SH1 modification on S1.

MATERIALS AND METHODS

Reagents

(*N*-1[1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl]iodacetamide (IASL) was from Aldrich (Milwaukee, WI). *N*-((2-(iodoacetoxy)ethyl)-*N*-methylamino)-7-nitrobenz-2-oxa-1,3-diazole (IANBD) was from Molecular Probes (Eugene, OR).

Proteins

Myosin and actin from back and leg muscles of rabbits were prepared according to Godfrey and Harrington (1970) and Spudich and Watt (1971), respectively. S1 from rabbit myosin was prepared by digestion of myosin filaments with α-chymotrypsin (Weeds and Pope, 1977). Heavy meromyosin was prepared according to Margossian and Lowey (1982). The concentrations of S1, HMM, and actin were determined spectrophotometrically by using the extinction coefficients of $E_{280}^{1\%} = 7.5\ cm^{-1}$, $E_{280}^{1\%} = 6.0\ cm^{-1}$, and $E_{290}^{1\%} = 11.5\ cm^{-1}$, respectively. The concentrations of SH1-modified and SH2-modified S1 and HMM were determined by using the Bradford protein assay (Bradford, 1976).

SH1 and SH2 modifications of S1 and HMM

SH1 modification was carried out according to Reisler (1982) in solutions containing between 10 and 20 μM S1 or HMM and a 20X molar excess of IASL. The reactions were carried out in 30 mM KCl and 20 mM Tris/HCl at pH 7.5, at 0°C, over 1 hour. SH2 modification was performed as described by Ajtai and Burghardt (1989) with some modifications (Root and Reisler, 1992) in solutions containing 10 μM S1 or 5 μM HMM, 30 μM F-actin, 4.0 mM Mg-ADP, 20 μM IANBD, 30 mM KCl, and 20 mM Tris/HCl (pH 8.0), at 0°C overnight. The SH2-modified S1 and HMM were separated from F-actin by ultracentrifugation in the presence of 3.0 mM Mg-ATP and 150 mM KCl. The extent of SH1 and SH2 modifications was determined by measuring the K^+-EDTA and Ca^{2+}-ATPase activities of S1 and HMM (Reisler, 1982; Ajtai and Burghardt, 1989). Typically, a 90–98% modified S1 and HMM were used in our experiments unless stated otherwise. More extensive labeling of myosin heads was avoided, because it required higher reagent concentrations and longer modification times, which could increase the probability of nonspecific modifications.

ATPase activities

The ATPase activities of S1 and HMM were measured at 37°C (Ca^{2+}- and K^+-EDTA-ATPase) and 20°C (Mg^{2+}-ATPase), under steady-state conditions, using the Malachite Green phosphate determination assay (Kodama et al., 1986). The Ca^{2+}-ATPase and K^+-EDTA-ATPase assay solutions contained 30 mM Tris/HCl (pH 7.5), 0.5 M KCl, and either 5.0 mM $CaCl_2$ or 5.0 mM EDTA. Mg^{2+}-ATPase measurements were done in solutions containing 10 mM PIPES (pH 7.0), 10 mM KCl, 3.0 mM $MgCl_2$, and 3.0 mM ATP in the presence and absence of F-actin (between 3 and 60 μM).

In vitro motility assays

In vitro motility assays were performed at 25°C as described elsewhere (Bobkov et al., 1997). In the assays with unregulated actin, modified HMM or mixtures of modified HMM and unmodified HMM were used with a total HMM concentration set at 0.3 mg/ml. Movement of filaments was initiated with solutions containing 0.4% methyl cellulose, 25 mM MOPS (pH 7.4), 25 mM KCl, 2.0 mM $MgCl_2$, 1.0 mM EGTA, 10 mM dithiothreitol, 1.0 mM ATP, and an oxygen-scavenging system. In the analysis of actin filament motility, filaments were considered to move uniformly using criteria described previously (Homsher et al., 1996).

Motility data obtained on mixtures of unmodified HMM and either IASL-HMM or IANBD-HMM were fitted using Eq. 1 (Cuda et al., 1997)

$$V = g_f g_s h[(1 + \sigma(\eta - 1)]^{1/2}/\{2[(1 - \sigma)g_s^2 + \sigma\eta g_r^2]\}^{1/2}, \quad (1)$$

where V is the sliding speed of actin filaments, g_r and g_s are the detachment rate constants of fast and slow cycling myosin, respectively, σ is the fraction of slow cycling myosin, η is the ratio of elastic force constant of the slow myosin to that of the fast myosin, and h is the displacement of the myosin head during a single power stroke. h and η were allowed to float freely in the fitting of data to Eq. 1. Fast and slow cycling myosins were equated with unmodified and modified HMM, respectively.

The assays with regulated actin were performed using unmodified HMM (0.3 mg/ml). Reconstitution of regulated thin filaments was carried out by incubating overnight on ice a mixture of 2.0 μM rhodamine-phalloidin-labeled F-actin with 0.5 μM bovine cardiac or skeletal tropomyosin and 0.5 μM bovine cardiac troponin in a buffer containing 4.0 mM imidazole/HCl at pH 7.1, 2.0 mM $MgCl_2$, and 1.0 mM dithiothreitol. Tropomyosin and troponin were a generous gift from Dr. Larry S. Tobacman. The 1.0 μM SH2-modified S1 or unmodified S1 was added to this solution in the motility activation experiments. Movement of filaments was initiated with solutions containing the same components as in the assays with unregulated actin except for the addition of 0.1 μM tropomyosin and 0.1 μM troponin and calcium to pCa 5, 7, and 8 levels. Tropomyosin and troponin were included in the assay solution to stabilize the regulated actin at the low protein concentration used in these experiments (Homsher et al., 1996).

Unmodified HMM and modified S1 and HMM were pre-spun before motility experiments with actin and ATP to remove damaged heads as

described before (Homsher et al., 1996). However, it should be noted that this procedure had no effect on the activation of regulated actin by modified S1 in the motility assays. Similar activation was observed with pre-spun and unspun modified S1.

F-actin binding

The binding of S1 and SH2-modified S1 to F-actin was measured using co-sedimentation assays (Miller and Reisler, 1995). The assay solutions contained S1, between 5.0 and 30 μM, 4.0 μM phalloidin-stabilized F-actin, 3 mM $MgCl_2$, 10 mM KCl, 10 mM PIPES (pH 7.0), and, in the case of weak binding, 3 mM ATP. The samples were centrifuged at room temperature in a Beckman airfuge at 140,000 $\times$ g for 10 min. Resuspended pellets and supernatants were examined on SDS-PAGE (Laemmli, 1970). The intensities of actin and S1 Commassie-Blue-stained bands were quantified by using a Biomed Instruments softlaser densitometer (Fullerton, CA). The values for the equilibrium dissociation constant (K_d) of S1 from actin were obtained by fitting the data to Eq. 2:

$$S/A = [(A + S + K_d) - \{(A + S + K_d)^2 - 4AS\}^{1/2}]/2A, \quad (2)$$

where S/A is the molar ratio of bound S1 to actin and A and S are the actin and S1 concentrations, respectively.

RESULTS

In vitro motility of actin filaments over the mixtures of unmodified and modified HMM

It was shown before that SH1 and SH2 modifications disrupted the ability of myosin to propel actin filaments in the in vitro motility assays (Root and Reisler, 1992; Marriott and Heidecker, 1996; Bobkov et al., 1997). To shed more light on the behavior of SH1- and SH2-modified myosin in the in vitro motility assays, we employed an approach used before by Cuda and colleagues (1997). Specifically, we measured the motility of unregulated actin filaments driven by mixtures of unmodified HMM and either SH1- or SH2-modified HMM. The results of such measurements are presented in Fig. 2. IASL-HMM (SH1-modified) and IANBD-HMM (SH2-modified) showed similar behavior in mixtures with unmodified HMM; the speed of actin filaments decreased in the same manner upon increase in the fraction of modified HMM. The speed of actin filaments approached zero in mixtures of 10% unmodified HMM and 90% of either IASL-HMM or IANBD-HMM.

Similar experiments were performed before on mixtures of fast-cycling myosin and either slow-cycling or noncycling myosins (Cuda et al., 1997). The slow-cycling and noncycling myosins inhibited motility of the fast-cycling myosin exerting a load on actin filaments. The slow-cycling myosins loaded actin in the strongly bound state, and the plots of the dependence of sliding speed of actin on the fraction of the fast-cycling myosin had concave shapes for these myosins. The noncycling myosins loaded actin in the weakly bound state, and the plots of the dependence of sliding speed of actin on the fraction of the fast-cycling myosin had convex shapes for these myosins. Our motility data for the mixtures of unmodified HMM and either IASL-HMM or IANBD-HMM (Fig. 2) are similar to the concave

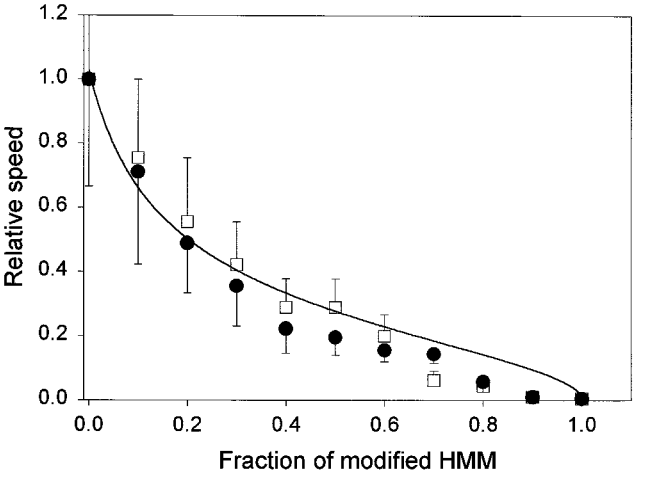

FIGURE 2 Relative sliding speeds of actin filaments driven by the mixtures of unmodified HMM and either SH1-modified HMM (□) or SH2-modified HMM (●). Speeds are shown relative to the speed of unmodified HMM. The bars indicate standard deviations. Fitting the data to Eq. 1 (only the fit for SH1-modified HMM is shown) yielded similar values for the ratios of detachment rate of unmodified HMM to that of modified HMM for IASL-HMM and IANBD-HMM ($g_r/g_s \approx 23$).

pattern shown by Cuda et al. (1997) for the mixtures of fast-cycling myosin with slow-cycling myosins. Therefore, we fitted our data using Eq. 1, which describes the behavior of mixtures of fast-cycling and slow-cycling myosins. As can be seen in Fig. 2, the calculated curve fits the experimental data reasonably well except for the lower part of the curve. There are two possible reasons for the deviation of the data from the curve. First, speed values for the motility of actin filaments over mixtures containing $\geq$70% of the modified HMM were less accurate because only a small fraction of actin filaments moved smoothly under these conditions. Second, the model of Cuda et al. (1997) may not describe well the behavior of modified heads. If the deviations from the calculated curve can be indeed attributed to experimental inaccuracy, then the fit of these data to Eq. 1 provides an estimate of the drag-stroke detachment rates for the modified HMMs (Cuda et al., 1997). The calculated ratios of the detachment rate of unmodified HMM to that of modified HMM ($g_r/g_s \approx 23$) were similar for IASL-HMM and IANBD-HMM. This suggests that the load exerted by the modified HMMs in the motility assays may be due to the much slower detachment rate of the modified HMMs than that of unmodified HMM.

In vitro motility with regulated actin and SH2-modified S1

We have shown before (Bobkov et al., 1997) that SH1-modified myosin heads can activate regulated actin. Here, we tested the effect of SH2 modification on activation properties of S1 in the in vitro motility assays. As before (Bobkov et al., 1997), to eliminate load due to the modified heads, IANBD-S1 was added to actin in solution instead of

being adsorbed to the coverslips. The results of such experiments are shown in Fig. 3. Open bars represent the movement of regulated actin (i.e., actin complexed with troponin and tropomyosin) propelled by unmodified HMM. This system was fully regulated by [Ca]; the speed and the fraction of filaments that moved declined with the decrease in [Ca] and reached zero at pCa 8. Addition of IANBD-S1 increased the sliding speed and the fraction of actin filaments that moved (Fig. 3, black bars). The activation effect was larger at pCa 7 and 8, when actin filaments were switched off, either partially or completely. Importantly, when the same amount of unmodified S1 was added to the assay solution instead of IANBD-S1, it had no effect on the motility (Fig. 3, gray bars). The activation effect of SH1-modified S1, which we observed before (Bobkov et al., 1997), was similar to the effect of SH2-modified S1 described here. The likely mechanism of this activation is that the binding of the modified S1s (but not that of unmodified S1 at the same concentration) switches on regulated actin. This increases the interaction of unmodified HMM heads with actin resulting in the increase in the fraction of filaments moving and in their sliding speeds.

Thus, it appears that both SH1 and SH2 modifications enhance the activation properties of S1 in a similar manner.

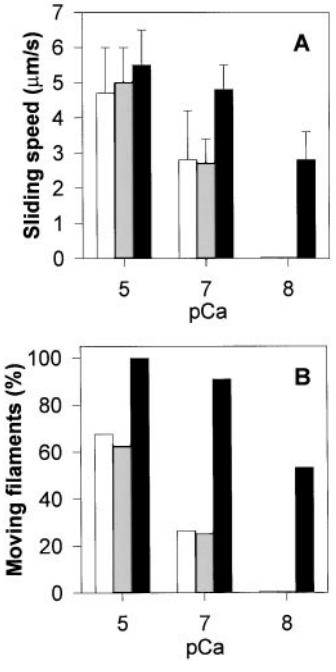

FIGURE 3 Sliding speeds (*A*) and the fraction of actin filaments (*B*) that moved uniformly in the in vitro motility assays with regulated actin. Open bars, actin filaments driven by unmodified HMM; gray bars, same as for the open bars except for the presence of 1.0 μM unmodified S1 in the assay solution; black bars, same as for the open bars except for the presence of 1.0 μM IANBD-S1 in the assay solution. Standard deviations are shown for all speed measurements.

ATPase activities

It is known that, although both SH1 and SH2 modifications inhibit the EDTA-ATPase of myosin equally well, the effects of these modifications on Ca-ATPase activities are different. Ca-ATPase of S1 is activated strongly by SH1 modification and is left unchanged by SH2 modification (Reisler et al., 1974a; Reisler, 1982). The effect of SH2 modification on basal Mg-ATPase activity of S1 has not been examined yet. It was shown that SH2 modification inhibits actin-activated ATPase of myosin (Root and Reisler, 1992), but the V_{max} and K_m values for SH2-modified S1 have not been reported so far. Table 1 presents a comparison of the effects of SH1 and SH2 modifications on the Mg-ATPase of S1 and its activation by actin. The first striking observation is that SH1 and SH2 modifications have opposite effects on the basal Mg-ATPase of S1. SH1 modification activates Mg-ATPase ~7-fold whereas SH2 modification inhibits it ~4-fold. The phosphate release step is a rate-limiting step in the Mg-ATPase cycle of S1, and the activation of Mg-ATPase of S1 by SH1 modification is mostly due to the acceleration of this step (Sleep et al., 1981). It is likely that the inhibition of basal Mg-ATPase of S1 by SH2 modification is mostly due to inhibition of the phosphate release step. Both SH1 and SH2 modifications decreased the V_{max} value for the S1 actin-activated ATPase. Interestingly, IASL-S1 lost almost completely actin activation (~2-fold) whereas IANBD-S1 retained a notable level of such activation (~20-fold) compared with unmodified S1 (~150-fold). Both modifications decreased the K_m value for S1 actin-activated ATPase; SH1 to a greater extent than the SH2 modification. However, the so-called enzymatic efficiency (V_{max}/K_m) was closer to that of the native protein for IASL-S1 than for IANBD-S1.

Actin binding

The load on unregulated actin filaments and the activation of the regulated actin produced by SH1- and SH2-modified heads in the in vitro motility assays imply that the actomyosin interactions are altered in some way by both modifications. We showed before that SH1 modification had no effect on the strong binding and slightly reduced the weak binding of S1 to actin (Bobkov et al., 1997). Here, we measured the strong and weak binding of SH2-modified S1 to actin (Table 2). IANBD-S1, similar to IASL-S1, had only a slightly reduced weak binding to actin. On the other hand, SH2 modification decreased by ~15-fold the strong binding of S1 to actin, whereas SH1 modification had no effect on such a binding.

DISCUSSION

Atomic resolution structures of chicken skeletal S1 and of *Dictyostelium* myosin S1 (S1dC) complexes with nucleotides, and nucleotide and phosphate analogs (Fisher et al., 1995; Rayment et al., 1993) confirmed the indications of

TABLE 1 Actin-activated and basal Mg-ATPase activities of SH1 (IASL-S1) and SH2-modified (IANBD-S1) S1

Protein	Mg-ATPase V_0 (s^{-1})	Actin-activated ATPase V_{max} (s^{-1})	K_m (μM)	V_{max}/K_m	Activation V_{max}/V_0
S1	0.056 ± 0.005	8.3 ± 1.7	30.0 ± 7.0	0.28	148
IANBD-S1	0.015 ± 0.001	0.35 ± 0.05	8.4 ± 0.8	0.042	23
IASL-S1	0.40 ± 0.04	0.93 ± 0.02	2.0 ± 0.2	0.47	2.3

The ATPase activities were measured at 20°C in 10 mM PIPES (pH 7.0), 10 mM KCl, 3.0 mM $MgCl_2$, and 3.0 mM ATP. Actin-activated ATPase assays contained also between 3.0 and 60 μM F-actin. The K_m and V_{max} values were obtained by fitting the data to the Michaelis-Menten equation. Each value is an average of at least three independent measurements. Data for IASL-S1 are taken from Bobkov et al. (1997).

many solution studies on the key role of the SH1-SH2 helix in myosin function. In particular, the transition between the Mg-ADP·BeF_x and Mg-ADP·V_i structures of S1dC, which is believed to model the transition between the Mg-ATP and Mg-ADP·P_i complexes of S1, involved changes in the switch II loop, the lower 50K region, and in the orientation of the SH2 group and, consequently, the pivoting of the SH1-SH2 helix (Fisher et al., 1995; Smith and Rayment, 1996). The location of the SH2 in the atomic structure of S1 and the above observed changes in SH2 environment in the Mg-ADP·BeF_x and Mg-ADP·V_i complexes of S1dC suggest its involvement in signal transduction on S1 from the ATP and actin sites to the mechanically important lever-arm region, i.e., the light-chain-binding domain.

Despite these obvious reasons for the interest in SH2, and in contrast to the many studies on SH1 modifications of S1, few results are available on SH2-altered myosin. Previous fluorescent (Hiratsuka, 1992; Phan et al., 1996) and cross-linking experiments (Rajasekharan et al., 1989) showed nucleotide-specific changes around SH2. Substitutions of SH2 (cysteine 678 on *Dictyostelium* myosin) for serine, alanine, threonine, and most of all, for glycine inhibited the sliding of actin filaments over such mutant myosin (Suzuki et al., 1997). Although these and other results are important, they do not provide a direct comparison of the SH2 and SH1 regions and the consequences of their manipulation for myosin functions.

The results of this study reveal some catalytic differences between SH1- and SH2-labeled S1s. These include opposite effects of such modifications on the basal Mg-ATPase (V_0) of S1 (activation and inhibition of the ATPase for SH1- and SH2-labeled S1, respectively) and, related to that, significant differences in the activation of S1 ATPase by actin (V_{max}/V_0). Moreover, the strong binding of S1 to actin appears to be unchanged for IASL-S1 although it is decreased almost 20-fold for IANBD-S1. On the other hand, both SH1 and SH2 modifications have no effect on the weak binding of S1 to actin, and both inhibit greatly the V_{max} of acto-S1 ATPase.

TABLE 2 Equilibrium dissociation constants (K_d) for the complexes of actin with SH1 (IASL-S1) and SH2-modified (IANBD-S1) S1

	K_d (μM)	
	No nucleotides	+ATP
S1	0.03 ± 0.01	33 ± 9
IANBD-S1	0.51 ± 0.26	42 ± 7
IASL-S1	0.03 ± 0.01	47 ± 12

Dissociation constants for unmodified S1 and IANBD-S1 were determined using co-sedimentation assays. Assay solutions contained S1, between 5.0 and 30 μM, 4.0 μM phalloidin-stabilized F-actin, 10 mM PIPES (pH 7.0), 10 mM KCl, and 3 mM $MgCl_2$. The weak binding was measured in the presence of 3.0 mM ATP. At least three separate determinations were made for each K_d value. Data for IASL-S1 are taken from Bobkov et al. (1997).

The above differences in the properties of SH1- and SH2-labeled S1, although not predictable, can be easily rationalized by assuming that the SH1-SH2 helix does not necessarily change as a single cooperative unit. In such a scenario, probes attached to SH2 and SH1 may have different effects on the local structure, flexibility, and mobility of the SH2 site and, consequently, via the switch II loop, on the catalytic events on S1. This idea is supported by recent mutational studies of Kinose et al. (1996) and Patterson et al. (1997) in which substitutions of Gly-680 (i.e., Gly-699 in skeletal myosin, next to SH2) and Gly-691 (i.e., Gly-710 in skeletal myosin, close to SH1) had similar, (i.e., opposing) effects on the basal Mg-ATPase of myosin to those shown for IASL-S1 and IANBD-S1 in this work.

Strikingly and importantly, the mechanical consequences of SH1 and SH2 modifications are virtually identical. Completely modified proteins do not move actin in the in vitro motility assays, and they introduce similar load into such assays in mixtures of modified and unmodified HMM. Both SH1- and SH2-modified HMMs have ~23-fold slower detachment rates from actin than the unmodified HMM if the Cuda et al. (1997) model is adopted for the analysis of our motility data. However, on their own, such slow detachment rates do not explain the loss of myosin's mechanical function; myosins with even slower detachment rates can move actin filaments (Cuda et al., 1997). It is also difficult to justify the mechanical inactivation of IANBD-HMM by changes in actin activation of Mg-ATPase. Although such a connection is valid for IASL-S1, with $V_{max}/V_0 \approx 2.3$, the larger actin activation of IANBD-S1 Mg-ATPase by actin, $V_{max}/V_0 \approx 23$, does not preclude the mechanical function for SH2-modified myosin. In this sense, SH2 modification achieves a greater uncoupling between the lever-arm and catalytic domains on S1 than the SH1 labeling.

The common denominator of both SH1 and SH2 modifications is most likely the change in the flexibility/mobility

of the corresponding parts of the SH1-SH2 helix. How extensive is the change may depend on the nature of the probe that is attached at these sites. It is also not clear yet whether these changes involve the reorientation of the SH2 or SH1, a partial immobilization of reactive SH groups, or just the opposite, a local unfolding of the helix. Support for flexibility-based explanations comes from two sources. Specific nucleotide effects on SH2- and SH1-attached probes (Hiratsuka, 1992; Phan et al., 1996) do not indicate an unfolded environment. More importantly, the replacement of Gly-699 on myosin (or its *Dictyostelium* counterpart) brought the actin motion almost to a halt (Kinose et al., 1996; Patterson et al., 1997), and that of Gly-710 also decreased significantly the in vitro motion of actin. A tempting hypothesis is that both the mutations and SH1 and SH2 modifications alter the flexibility of the corresponding region in the SH1-SH2 helix. Accordingly, the mechanical function of the lever arm can be disrupted by changes in the flexibility at either the SH1 or SH2 site. The proposed swinging of the lever arm of S1 relative to the catalytic domain, with the pivot point located in the vicinity of the SH1-SH2 helix (Uyeda et al., 1996; Whittaker et al., 1995; Suzuki et al., 1997) appears to depend on the structural integrity and flexibility at both SH1 and SH2. Clearly, although such a speculative explanation of our results can account for different catalytic but similar mechanical results of SH1 and SH2 modifications, it does not clarify the similar activation of regulated actin filaments by IASL-S1 and IAMSD-S1. The mechanistic explanation of this effect awaits further investigation.

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REFERENCES

Ajtai, K., and T. P. Burghardt. 1989. Fluorescent modification and orientation of myosin sulfhydryl 2 in skeletal muscle fibers. *Biochemistry*. 28:2204–2210.

Bobkov, A. A., E. A. Bobkova, E. Homsher, and E. Reisler. 1997. Activation of regulated actin by SH1-modified myosin subfragment 1. *Biochemistry*. 36:7733–7738.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Burke, M., and E. Reisler. 1977. Effect of nucleotide binding on the proximity of the essential sulphydryl groups of myosin: chemical probing of movement of residues during conformational transitions. *Biochemistry*. 16:5559–5563.

Cuda, G., E. Pate, R. Cooke, and J. R. Sellers. 1997. In vitro actin filament sliding velocities produced by mixtures of different types of myosin. *Biophys. J.* 72:1767–1779.

Fisher, A. J., C. A. Smith, J. B. Thoden, R. Smith, K. Sutoh, H. M. Holden, and I. Rayment. 1995. X-ray structures of the myosin motor domain of *Dictyostelium discoideum* complexed with MgADP-BeF_x and MgADP-AlF_4^-. *Biochemistry*. 34:8960–8972.

Godfrey, J., and W. F. Harrington. 1970. Self-association in the myosin system at high ionic strength: sensitivity of the interaction to pH and ionic environment. *Biochemistry*. 9:886–893.

Golitsina, N. L., A. A. Bobkov, I. V. Dedova, D. A. Pavlov, O. P. Nikolaeva, V. N. Orlov, and D. I. Levitsky. 1996. Differential scanning calorimetric study of the complexes of modified myosin subfragment 1 with ADP and vanadate or beryllium fluoride. *J. Muscle Res. Cell Motil.* 17:475–485.

Hiratsuka, T., 1992. Movement of Cys-697 in myosin ATPase associated with ATP hydrolysis. *J. Biol. Chem.* 267:14941–14948.

Homsher, E. H., B. Kim, A. Bobkova, and L. S. Tobacman. 1996. Calcium regulation of thin filament. *Biophys. J.* 70:1881–1892.

Kodama, T., K. Fukui, and K. Kometani. 1986. The initial phosphate burst in ATP hydrolysis by myosin and subfragment-1 as studied by a modified malachite green method for determination of inorganic phosphate. *J. Biochem.* 99:1465–1472.

Kinose, F., S. X. Wang, U. S. Kidambi, C. L. Moncman, and D. A. Winkelmann. 1996. Glycine 699 is pivotal for the motor activity of skeletal muscle myosin. *J. Cell Biol.* 134:895–909.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227:680–685.

Margossian, S. S., and S. Lowey. 1982. Preparation of myosin and its subfragments from rabbit skeletal muscle. *Methods Enzymol.* 85:55–72.

Marriott, G., and M. Heidecker. 1996. Light-directed generation of the actin-activated ATPase. Activity of caged heavy meromyosin. *Biochemistry* 35:3170–3174.

Miller, C. J., and E. Reisler 1995. Role of charged amino acid pairs in subdomain 1 of actin in interactions with myosin. *Biochemistry* 34: 2694–2700.

Mulhern, S. A., and E. Eisenberg. 1978. Interaction of spin-labeled and N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid SH1-blocked heavy meromyosin and myosin with actin and adenosine triphosphate. *Biochemistry*. 17:4419–4425.

Ostap, E. M., H. D. White, and D. D. Thomas. 1993. Transient detection of spin-labeled myosin subfragment 1 conformational states during ATP hydrolysis. *Biochemistry*. 32:6712–6720.

Patterson, B., K. M. Ruppel, Y. Wu, and J. A. Spudich. 1997. Cold-sensitive mutants G680V and G691C of *Dictyostelium* myosin II confer dramatically different biochemical defects. *J. Biol. Chem.* 272: 27612–27617.

Phan, B. C., P. Cheung, W. F. Stafford, and E. Reisler. 1996. Complexes of myosin subfragment-1 with adenosine diphosphate and phosphate analogs: probes of active site and protein conformation. *Biophys. Chem.* 59:341–349.

Rajasekharan, K. N., M. Mayadevi, and M. Burke. 1989. Studies of ligand-induced conformational perturbations in myosin subfragment 1. *J. Biol. Chem.* 25:10810–10819.

Rayment, I., W. R. Rypniewski, K. Schmidt-Base, R. Smith, D. R. Tomchick, M. M. Benning, D. A. Winkelmann, G. Wesenberg, and H. M. Holden. 1993. Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science*. 261:50–58.

Reisler, E. 1982. Sulfhydryl modification and labeling of myosin. *Methods Enzymol.* 85:84–93.

Reisler, E., M. Burke, and W. F. Harrington. 1974a. Cooperative role of two sulfhydryl groups in myosin. *Biochemistry*. 13:2014–2022.

Reisler, E., M. Burke, S. Himmelfarb, and W. F. Harrington. 1974b. Spatial proximity of the two essential sulfhydryl groups of myosin. *Biochemistry*. 13:3837–3840.

Root, D. D., and E. Reisler. 1992. Cooperativity of thiol-modified myosin filaments: ATPase and motility assays of myosin function. *Biophys. J.* 63:730–740.

Sleep, J. A., K. M. Trybus, K. A. Johnson, and E. W. Taylor. 1981. Kinetic studies of normal and modified heavy meromyosin and subfragment 1. *J. Muscle Res. Cell Motil.* 2:373–399.

Smith, C. A., and I. Rayment. 1996. X-ray structure of the magnesium(II)-ADP-vanadate complex of the *Dictyostelium discoideum* myosin motor domain to 1.9 Å resolution. *Biochemistry*. 35:5404–5417.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction: biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4876.

Suzuki, Y., R. Ohkura, S. Sugiura, R. Yasuda, K. Kinoshita Jr., M. Tanokura, and K. Sutoh. 1997. Modulation of actin filament sliding by mutations of the SH2 cysteine in *Dictyostelium* myosin II. *Biochem. Biophys. Res. Commun.* 234:701–706.

Uyeda, T. Q. P., P. D. Abramson, and J. Spudich. 1996. The neck region of the myosin motor domain acts as a lever arm to generate movement. *Proc. Natl. Acad. Sci. U.S.A.* 93:4459–4464.

Weeds, A. G., and B. Pope. 1977. Studies on the chymotryptic digestion of myosin: effects of divalent cations on proteolytic susceptibility. *J. Mol. Biol.* 111: 129–157.

Wells, J. A., C. Knoeber, M. C. Sheldon, M. M. Werber, and R. G. Yount. 1980. Cross-linking of myosin subfragment 1: nucleotide-enhanced modification by a variety of bifunctional reagents. *J. Biol. Chem.* 255: 11135–11140.

Wells, J. A., and R. G. Yount. 1980. Reaction of 5,5′-dithiobis(2-nitrobenzoic acid) with myosin subfragment one: evidence for formation of a single protein disulfide with trapping of metal nucleotide at the active site. *Biochemistry*. 19:1711–1717.

Whittaker, M., Wilson-Kubalek, E. M., Smith, J. E., Faust, L., Milligan, R. A., and H. L. Sweeney. 1995. A 35-Å movement of smooth muscle myosin on ADP release. *Nature*. 378:748–751.