Cooperativity of thiol-modified myosin filaments

ATPase and motility assays of myosin function

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ABSTRACT The effects of chemical modifications of myosin's reactive cysteines on actomyosin adenosine triphosphatase (ATPase) activities and sliding velocities in the in vitro motility assays were examined in this work. The three types of modifications studied were 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole labeling of SH_2 (based on Ajtai and Burghart. 1989. *Biochemistry*. 28:2204–2210.), phenylmaleimide labeling of SH_1, and phenylmaleimide labeling of myosin in myofibrils under rigor conditions. Each type of modified myosin inhibited the sliding of actin in motility assays. The sliding velocities of actin over copolymers of modified and unmodified myosins in the motility assay were slowest with rigor-modified myosin and most rapid with SH_2-labeled myosin. The actin-activated ATPase activities of similarly copolymerized myosins were lowest with SH_2-labeled myosin and highest with rigor-modified myosin. The actin-activated ATPase activities of myosin subfragment-1 obtained from these modified myosins decreased in the same linear manner with the fraction of modified heads. These results are interpreted using a model in which the sliding of actin filaments over myosin filaments decreases the probability of myosin activation by actin. The sliding velocity of actin over monomeric rigor-modified myosin exceeded that over the filamentous form, which suggests for this myosin that filament structure is important for the inhibition of actin sliding in motility assays. The fact that all cysteine modifications examined inhibited the actomyosin ATPase activities and sliding velocities of actin over myosin poses questions concerning the information about the activated crossbridge obtained from probes attached to SH_1 or SH_2 on myosin.

INTRODUCTION

Adenosine 5'-triphosphate (ATP)[1] drives cyclic interactions between actin and myosin. In vitro motility analysis and solution studies have shown that these interactions can produce sliding of actin filaments over surfaces covered with purified myosin and elevate the adenosine triphosphatase (ATPase) activity of myosin. The mechanism by which the free energy in ATP is converted to force production and contraction in muscle, however, remains unclear. The understanding of this mechanism and of the underlying actomyosin interactions requires coordination of studies in solution and in fibers. Measurements in the latter system normally use spin-labels or fluorescent probes to monitor conformational changes in the myosin head induced by ATP (for reviews, see Cooke, 1986; Thomas, 1987). However, in each case, the effects of introducing such probes on the target protein must be examined in solution with the purified proteins.

Two reactive cysteines on the myosin heavy chain, SH_1 and SH_2, have been labeled specifically in the fiber for the purpose of examining structural changes in the myosin head during muscle contraction (Duke et al., 1976; Ajtai and Burghart, 1989). Although characterization of the effects of SH_2 modification on myosin function in solution has been limited (Reisler et al., 1974), several studies are available on the effects of SH_1 modification (Sekine and Kielley, 1964; Silverman et al., 1972; Mulhern and Eisenberg, 1976, 1978; Root et al., 1991). Although it was shown that labeling SH_1 inhibited the actin-activated ATPase activity of myosin fragments (Silverman et al., 1972; Mulhern and Eisenberg, 1976, 1978; Svensson and Thomas, 1986), this inhibition in whole myosin by labeling SH_1 was more controversial. Studies in which a high proportion of the SH_1 cysteines was modified found inhibition of the actomyosin ATPase activity (Silverman et al., 1972; Mulhern and Eisenberg, 1978; Titus et al., 1989; Root et al., 1991), but when a lesser fraction of the SH_1 cysteines was modified, no apparent inhibition of actomyosin ATPase activity was detected (Seidel, 1973; Mendelson et al., 1975; Thomas et al., 1980; Thomas and Cooke, 1980; Fajer et al., 1988). We previously showed that despite its impaired actin-activated ATPase, the SH_1-modified myosin induced a "catalytic cooperativity" in copolymers with unmodified myosin, i.e., it increased the actomyosin ATPase of unmodified myosin. The cooperativity occurred only in filamentous form of myosin. It was implied from these results that the impaired function of the modified heads was masked by intrafilament interactions. The effect of SH_1 modification on the ability of modified myosin to generate sliding in the in vitro motility assay, however, remained to be tested. A preliminary

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[1] *Abbreviations used in this paper:* ATP, adenosine 5'-triphosphate; ATPase, adenosine triphosphatase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; EGTA, ethyleneglycol - bis(aminoethyl ether)-*N,N'*-tetraacetic acid; HMM, heavy meromyosin; IAA, iodoacetamide; IANBD, 4-[N-[(iodoacetoxy)ethyl]-N-methylamino]-7-nitrobenz-2-oxa-1,3-diazole; PM, phenylmaleimide; S-1, myosin subfragment-1; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; V_{max}, maximum velocity.

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report indicated that SH_1 labeling of myosin subfragment-1 (S-1) and heavy meromyosin (HMM) inhibited their ability to produce sliding in the in vitro motility assays (Chaen et al., 1991).

It has not been possible to specifically modify cysteines on the myosin heavy chain other than SH_1 and SH_2. The use of rigor conditions for labeling of muscle fibers with *p*-nitrophenylenedimaleimide and *N*-phenylmaleimide has been introduced to reduce the modification of SH_1 and increase that of other cysteines (Barnett and Schoenberg, 1992). The results of such studies suggested that the modification of cysteines other than SH_1 could influence fiber stiffness (Barnett and Schoenberg, 1992). Inhibition of SH_1 modification in the muscle fiber by KI and KF also has been used to study the effect of nonspecific labeling of cysteines, which reportedly decreased fiber tension (Crowder et al., 1983). One question that such studies raise is whether the observed effects are caused by modifications on myosin or on other proteins in the fiber.

This study compares the effects of modifications of SH_1, SH_2, and nonspecific thiols on myosin isolated from rigor-modified myofibrils on ATPase activities and sliding velocities of actin in the in vitro motility assays. In the actin-activated ATPase activity assays, S-1 obtained from the modified myosin was compared with myosin filaments to test for catalytic cooperativity in the modified filaments. In the in vitro motility assays, myosin was applied either as monomers or as filaments to the coverslip to correlate the sliding velocities of actin with the modification and assembly state of myosin. Each type of modification examined had unique effects on the behavior of filamentous myosin. At the limit of complete reactions, each modification impaired actomyosin ATPase activity and sliding in the motility assays. Myosin labeled on SH_2 did not induce catalytic cooperativity in actomyosin ATPase activity assays and showed the least inhibition of actin sliding over mixtures of labeled and unlabeled myosin. Greater inhibition of sliding velocities was produced by SH_1-labeled myosin. Rigor-modified myosin induced even greater catalytic cooperativity in actomyosin ATPase activity and greater inhibition of actin sliding velocities than did SH_1-labeled myosin.

MATERIALS AND METHODS

Reagents

Trypsin, soybean trypsin inhibitor, *N*-α-para-tosyl-L-lysine chloromethyl ketone-treated α-chymotrypsin, phenylmethylsulfonyl fluoride, papain, bis-tris(hydroxymethyl)-aminomethane (Tris), *N*-ethylmaleimide, ATP, 5,5′-dithiobis[2-nitrobenzoic acid], ethylene glycol bis(β-amino ethyl ether)-*N,N′*-tetracetic acid (EGTA), and β-mercaptoethanol were procured from Sigma Chemical Co. (St. Louis, MO). 4-[N-[(iodoacetoxy)ethyl]-N-methylamino]-7-nitrobenz-2-oxa-1,3-diazole (IANBD) was obtained from Molecular Probes (Eugene, OR). Dithiothreitol (DTT) and phenylmaleimide (PM) were purchased from Schwarz/Mann Biotech (Cleveland, OH) and Aldrich Chemical Co. (Milwaukee, WI). X-Omat AR X-ray film and [2,4,6-^{3}H]-phenylmaleimide were obtained from Eastman Kodak (Rochester, NY) and Amersham International (Amersham, England), respectively. 2,5-diphenoxazole and ethylenediaminetetraacetate (EDTA) were procured from Fisher (Fairlawn, NJ). The Bradford protein assay was purchased from Bio-Rad (Richmond, CA). Filtered (Millipore Corp., Bedford, MA) distilled water and analytical grade reagents were used in all experiments.

Proteins

Myofibrils were prepared from rabbit skeletal muscle as previously described (Duong and Reisler, 1989). Myosin was extracted from these myofibrils in a solution of (mM) 300 KCl, 10 ATP, 10 $MgCl_2$, 2 sodium pyrophosphate, and 10 imidazole, pH 7.0, and then centrifuged for 2 h at 4°C in a (model Ti50; Beckman Instruments, Inc., Fullerton, CA) at 35,000 rpm. S-1 was prepared from the myofibrils by digesting 10 mg/ml of myofibrils in 120 mM KCl, 2 mM EDTA, and 10 mM Tris, pH 8.0, with 0.2 mg/ml of α-chymotrypsin for 45 min at 22°C (Duong and Reisler, 1989). The cleaved myofibrils were extracted first as for myosin above, and then the supernatant was dialyzed for 1 d against 40 mM KCl and 10 mM imidazole, pH 7.0, and centrifuged for 2 h at 4°C in a Beckman It 50 rotor at 35,000 rpm. The concentration of the myofibrils was estimated by spectrophotometric readings in 5% (wt/vol) sodium dodecyl sulfate (SDS) using $E^{1\%}_{280nm} = 7.0\ cm^{-1}$ (Sutoh and Harrington, 1977).

Purified rabbit skeletal myosin was prepared as described before (Godfrey and Harrington, 1970). S-1 was obtained from the myosin with α-chymotrypsin and separated according to Weeds and Pope (1977). The concentrations of myosin and S-1 were measured spectrophotometrically by using $E^{1\%}_{280nm} = 5.5\ cm^{-1}$ in high salt for myosin (Godfrey and Harrington, 1970) and $E^{1\%}_{280nm} = 7.5\ cm^{-1}$ for S-1 (Wagner and Weeds, 1977). Rabbit skeletal muscle actin was extracted from acetone powder (Spudich and Watt, 1971). Its concentration was determined spectrophotometrically by using $E^{1\%}_{280nm} = 11.0\ cm^{-1}$ (West et al., 1967).

Labeling myosin and myofibrils

Specific labeling of SH_1 groups on the myosin head in solution has been described before (Burke and Reisler, 1977). Isolated myosin at 7 mg/ml was labeled with PM in 0.6 M KCl and 10 mM imidazole, pH 7.0, for 15 min at 4°C. The reaction was terminated with a 10-fold molar excess of β-mercaptoethanol over the modifying reagent. The extent of SH_1 modification was checked by Ca^{2+} and K^+ (EDTA) ATPase activities.

Rigor-modified myosin was extracted from myofibrils (2.0 mg/ml) modified under rigor conditions in the presence of 30 mM KCl, 2 mM EGTA, and 10 mM imidazole, pH 7.0, with concentrations of PM up to 0.5 mM (dissolved initially in dimethylformamide so that the final amount of organic solvent was <3%) for 30 min at 4°C. After the modification, myofibrils were washed and resuspended in 0.3 M KCl, 5 mM $MgCl_2$, 2 mM sodium pyrophosphate, 5 mM ATP, and 10 mM imidazole, pH 7.0, before centrifugation at 35,000 rpm for 1 h in a Beckman ultracentrifuge. The extent of modification was estimated from the K^+ (EDTA) ATPase activity of the extracted myosin.

The labeling of SH_2 with IANBD used a modified procedure of Ajtai and Burghart (1989). Purified myosin filaments (10 mg/ml) in 0.14 M KCl, 10 mM Tris, pH 8.0, were mixed with a threefold molar excess of purified actin (7 mg/ml) in G-buffer to which 5 mM $MgCl_2$ and 2 mM adenosine 5′-diphosphate were added. The actomyosin was incubated overnight at 4°C with varying concentrations of IANBD (up to fourfold molar excess). The reaction was stopped by adding 1 mM DTT. The myosin was separated from the actin by adding 0.3 M KCl, 2 mM sodium pyrophosphate, and 3 mM ATP, and centrifuging at 35,000 rpm for 1 h in a Beckman ultracentrifuge.

Concentrations of all modified proteins were determined by Bradford protein assays (Bradford, 1976), as modified by Bio-Rad according to the manufacturer's instructions.

Turbidometric assays of shortening

The extent of myofibrillar shortening was estimated by turbidity measurements on a continuously pumped suspension of myofibrils (0.1 mg/ml) in a spectrophotometer (model 25; Beckman) equipped with a sipper cell as previously described (Root et al., 1991; Maruyama and Kominz, 1969; Kominz, 1970).

ATPase assays

Ca^{2+} and K^+ (EDTA) ATPase assays were performed according to the methods of Kielly and Bradley (1956). The Mg^{2+} ATPase of myofibrils at 0.4 mg/ml was measured in (mM) 30 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 imidazole, pH 7.0, and 4 ATP at 25°C. Actin-activated ATPases for S-1 and myosin filaments were measured at 25°C in (mM) 2 $MgCl_2$, 10 imidazole, pH 7.0, 4 ATP, and at KCl concentrations as specified in the figure legends. Samples were incubated for 3–10 min and stopped by the addition of an equal volume of 10% trichloroacetic acid. At least 3 mM of unhydrolyzed ATP remained on termination of the assay; thus, no superprecipitation occurred. Denatured protein was removed by centrifugation. Determinations of the amount of inorganic phosphate released during the assays were made as previously described (Reisler, 1980). ATPase activity was expressed per mole of myosin heads (modified + unmodified).

Binding assay

Relative binding affinities of SH_1-modified, rigor-modified, and unmodified S-1 for actin were determined by 90° light scattering at 325 nm in a fluorolog spectrofluorimeter (SPEX Industries, Inc., Edison, NJ) at 25°C as previously described (Root et al., 1991). The light scattering varied linearly with the amount of S-1 added ($r^2 \geq 0.87$). Based on light scattering measurements of the fraction of actin bound to unmodified S-1 in the presence of ATP, a K_a of 8×10^3 M^{-1} was estimated. This value of K_a agrees with values between 2×10^3 M^{-1} and 2×10^4 M^{-1} determined by Chalovich and Eisenberg (1982) from sedimentation experiments in the presence of ATP at varying ionic strengths.

Thiol titrations

Thiol titrations were carried out to determine the extent of sulfhydryl modification of S-1 (Wells and Yount, 1980). Samples of S-1 (0.2 ml at 1 mg/ml) were mixed with 0.7 ml of denaturing solution (9 M urea, 10 mM EDTA, 0.1 M KCl, and 50 mM Tris, pH 8.0) for 30 min, and then 0.2 ml of the titration reagent (1 mM 5,5'-dithiobis[2-nitrobenzoic acid], 0.1 M KCl, and 10 mM $K_2HPO_4 \cdot 3H_2O$) was added, incubated for 15 min at 22°C, and read using $E_{412\,nm} = 1.36 \times 10^4$ M^{-1} (Ellman, 1959).

Fluorography

Myofibrils were labeled with [2,4,6-^{3}H]phenylmaleimide until partial or full loss of shortening was obtained. The myofibrils, chymotryptically-cleaved myofibrils, isolated S-1, tryptically-digested S-1, and S-1 partially cleaved by papain (Applegate and Reisler, 1983) were run on a 5–20% (wt/wt) polyacrylamide linear-gradient gels made according to the method of Laemmli (1970). Fluorography was performed with 2,5-diphenoxazole on X-Omat AR film (Eastman Kodak) as described previously (Bonner, 1984). A soft laser densitometer (model SL-504-XL; Biomed Instruments Inc., Fullerton, CA) equipped with an integrator and interfaced to an Apple IIe computer was used to quantify the bands on the fluorogram.

In vitro motility assay

The motility assays were performed essentially as described (Kron et al., 1991) with a few modifications. A fluorescent microscope (Orthoplan, E. Leitz Inc., Rockleigh, NJ) with a 200 W mercury bulb, built-in neutral density filter, and 63× oil immersion objective (N.A. = 1.3) were used to image the Rhodamine Phalloidin-labeled actin filaments. Phalloidin does not effect the K_m or maximum velocity (V_{max}) of actin-activated ATPase activities (Uyeda et al., 1990). The images were recorded on a videocassette recorder (model SLV-R5; Sony, Park Ridge, NJ) from a camera (model SIT68; Dage-MTI Inc., Michigan City, IN) after digital image processing with a processor (model ARGUS 10; Hamamatsu Corp., Bridgewater, NJ). Temperature was maintained at 25°C with a brass microscope stage and sleeve for the objective. Myosin was applied to nitrocellulose-coated (Kron et al., 1991) or siliconized (Harada et al., 1990) glass coverslips at 0.2 and 3–5 mg/ml, respectively. Experiments were repeated on both surfaces with no apparent differences in sliding velocities of actin over modified myosins relative to those over the control myosin. However, sliding velocities of actin over the control myosin were higher on the siliconized coverslips than on the nitrocellulose-coated ones (3.9 ± 0.7 vs. 2.0 ± 0.3 μM/s).

Great care was taken to remove ATP-insensitive myosin heads. Immediately before a motility assay, myosin was treated with a twofold molar excess of F-actin. This actin was removed by adding 0.3 M KCl, 2 mM sodium pyrophosphate, and 3 mM ATP and centrifuging at 35,000 rpm for 1 h in a Beckman ultracentrifuge to pellet F-actin and irreversibly-bound myosin heads (Kron et al., 1991). Myosin was then applied to the coated coverslip, and the excess myosin was washed away with assay buffer. To block any ATP-insensitive heads that might form during adsorption of myosin to the coverslip (Homsher et al., 1992), unlabeled F-actin (10 μM) was added to the coverslip and unbound F-actin was washed away with assay buffer [(mM) 25 KCl, 1 EGTA, 5 $MgCl_2$, 10 DTT, 25 imidazole, pH 7.0] containing 1 mM ATP and oxygen scavenging enzymes (0.1 mg/ml glucose oxidase, 0.018 mg/ml catalase, and 3 mg/ml glucose). A low salt assay buffer (25 mM KCl) was used, because high salt concentrations (0.1 M KCl) inhibited the sliding velocity on myosin filaments (data not shown) as was found by Harada et al. (1987).

Quantification of sliding velocities was achieved with a three-dimensional system (ExpertVision; Motion Analysis Corp., Santa Rosa, CA). Sliding velocities of all actin filaments tracked were averaged for statistical analysis. The velocities of typically ~100 filaments were averaged for each determination. Because the noise level of the tracking system increased in an approximately linear fashion with the frame rate selected (data not shown) (Homsher et al., 1992), frame rates were chosen that gave a noise level three to five times lower than the mean sliding velocity of the filaments (Homsher et al., 1992). When actin filaments from selected sequences were tracked manually with an ARGUS 10 image processor, nearly identical results were obtained as with the Motion Analysis system (data not shown).

RESULTS

Modifications of myosin

Rigor vs. relaxed modification on myofibrils

Modification of myofibrils under relaxed conditions with PM has been shown to result in relatively specific modification of the SH_1 thiol on myosin (Root et al., 1991). As expected for such a modification, a plot of K^+ (EDTA) ATPase activity versus the number of thiols modified on S-1 was nearly linear and extrapolated to a loss of one thiol on complete inactivation of S-1 (Root et al., 1991). On the other hand, modification with PM under rigor conditions is known to suppress SH_1 labeling (Duke et al., 1976; Barnett and Schoenberg, 1992). A similar plot of K^+ (EDTA) ATPase activity versus the number of thiols labeled on S-1 prepared from modified myofibrils indicates that between two and three thiols

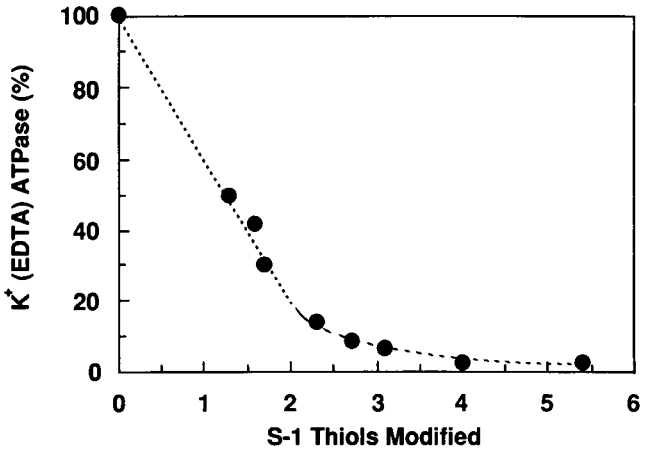

FIGURE 1 K^+ (EDTA) ATPase activity vs. the number of modified thiols on S-1. S-1 was extracted from myofibrils modified to varying extents with PM under rigor conditions. The extracted S-1 was subjected to 5,5'-dithiobis[2-nitrobenzoic acid] thiol titrations. The number of thiols blocked by PM is plotted on the x axis. The K^+ (EDTA) ATPase activity is shown on the y axis.

are modified on complete loss of this ATPase activity (Fig. 1).

The sites of PM labeling in rigor myofibrils were determined by fluorography of [2,4,6-^{3}H]phenylmaleimide-modified myofibrils run on SDS-polyacrylamide gel electrophoresis (data not shown). Most of the modifications were on actin, myosin, and some high molecular weight proteins, probably titin (Barnett and Schoenberg, 1992). Densitometric measurements of the fluorograms indicated that the label distribution on myosin was 37% on the rod, 37% on the alkali and regulatory light chains, and 26% on the tryptic 20 kD fragment of the heavy chain of S-1. Together with the thiol titrations of Fig. 1, this distribution indicates that the labeling of one to two thiols on the S-1 heavy chain is responsible for the loss of K^+ (EDTA) ATPase activity. The distributions of the radioactive probe were approximately the same whether the myofibrils were modified to 35 or 70% loss of K^+ (EDTA) ATPase activity (data not shown).

Within S-1, the modification site is normally deduced from ATPase measurements. The K^+ (EDTA) ATPase activity is abolished by labeling either SH_1 or SH_2 (Reisler et al., 1974). Assuming that only SH_1 and SH_2 modifications effect the K^+ (EDTA) ATPase activity and that they are the only accessible critical thiols on the heavy chain of S-1, the K^+ (EDTA) ATPase indicates the number of heads with at least one critical thiol modified. The Ca^{2+} ATPase activity is elevated by SH_1 modifications and inhibited by labeling both SH_1 and SH_2 groups. Specific modification of SH_2 has no effect on that activity (Reisler et al., 1974). In this study, specific modification of SH_1 groups with PM elevated the Ca^{2+} ATPase activity of myosin sixfold. The lower elevation of Ca^{2+} ATPase activity in rigor-modified myosin (about twofold) suggests that under rigor conditions, the reactivity of the SH_2 thiol has been increased relative to that of the SH_1 thiol.

Labeling with PM under rigor conditions, as opposed to relaxed conditions, has a strikingly different effect on the shortening of myofibrils. The inhibition of the extent of myofibril shortening by rigor modification is estimated in Fig. 2 by turbidity measurements. The comparison to similar turbidity measurements on myofibrils modified under relaxed conditions, when only SH_1 is labeled (Duke et al., 1976; Root et al., 1991), shows that the inhibition caused by rigor modifications cannot be attributed solely to SH_1 labeling. Whether the inhibition of myofibril shortening was caused by modifications on myosin or other proteins had to be determined by reconstituting the extracted myosin with purified actin for actomyosin ATPase and in vitro motility assays.

Specific labeling of SH_2 on myosin

The hydrophobic reagent IANBD was found to have a greater affinity for SH_2 groups than other thiol reagents (Ajtai and Burghart, 1989). By enhancing the reactivity of SH_2 relative to SH_1, Ajtai and Burghart (1989) achieved a rather specific modification of SH_2 in the fiber. A modification of their procedure enables a specific labeling of SH_2 on actomyosin in solution. The labeling of SH_2 was more specific at pH 8.0 than at pH 7.0 (data not shown). As shown in Fig. 3, the Ca^{2+} ATPase activity of SH_2-labeled myosin was unaltered even

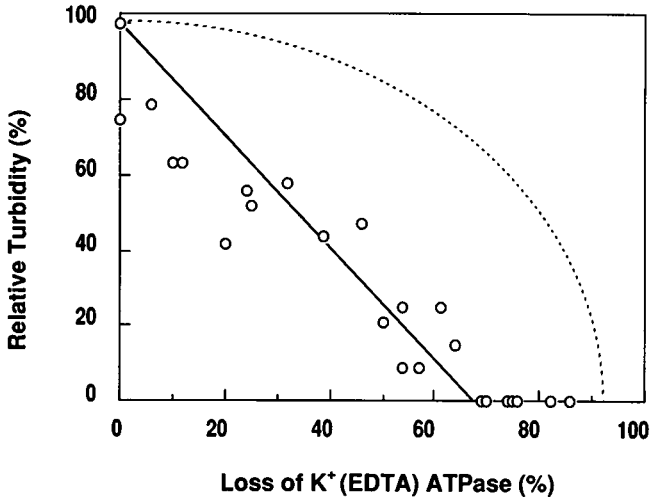

FIGURE 2 Loss of turbidity for relaxed vs. rigor-modified myofibrils. Myofibrils were modified to varying extents under rigor conditions with PM (O) and assayed for their ability to contract by using a turbidometric assay as described in the Materials and Methods. Myofibrils (0.1 mg/ml) were suspended in (mM) 30 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 10 imidazole, pH 7, to which 20 μM ATP was added to initiate the contraction at 22°C. The relative changes in optical density measured at 650 nm are plotted on the vertical axis. The loss of K^+ (EDTA) ATPase activities in the extracted myosin, which monitors the percentage of modified heads, is plotted on the horizontal axis. The dashed curve is taken from similar measurements made previously on relaxed-modified myofibrils (Root et al., 1991).

though its K^+ (EDTA) ATPase activity was almost completely inhibited. These data are consistent with the reported features of SH_2-modified S-1 (Reisler et al., 1974). For myosin modified to between 80 and 100% loss of K^+ (EDTA) ATPase activity, the amount of IANBD incorporated per myosin head divided by the fractional loss of K^+ (EDTA) ATPase activity was determined by absorbance to be 1.12 ± 0.09, using the method described by Ajtai and Burghart (1989). Hence, it appears that the modification of only one thiol is responsible for the observed effects. As reported by these authors, IANBD was incorporated almost exclusively into the 20-kD tryptic fragment of S-1 (data not shown). Samples could not be subjected to hydroxylamine cleavage or even boiled before application to the SDS-polyacrylamide gel electrophoresis because of a sensitive ester linkage in IANBD (Ajtai and Burghart, 1989). Further evidence that the IANBD modified SH_2 rather than SH_1 was that a secondary modification of IANBD-labeled myosin with an SH_1-specific reagent, iodoacetamide (IAA), abolished the Ca^{2+} ATPase activity of myosin (data not shown). As judged by the Ca^{2+} ATPase activity of IANBD-labeled myosin, the SH_1 groups on this protein were fully labeled after a 15-min reaction at 4°C with a 40-fold molar excess of IAA over S-1. Under the same conditions, as detected by loss of K^+ (EDTA) ATPase activity, only ~11% of the SH_1 groups were labeled by IAA on unmodified myosin (data not shown).

Actin-activated ATPase activities of modified myosins

The profile of actomyosin ATPase activities depended greatly on the type of modification used. As shown in Fig. 4, both SH_1 and rigor-modified myosins exhibit catalytic cooperativity, i.e., the V_{max} values for these proteins are not inhibited in proportion to the loss of K^+ (EDTA) ATPase activity (Root et al., 1991). However, in the same figure, SH_2-modified myosin loses actomyosin ATPase activity in proportion to the extent of its modification. The rigor-modified myosin shows even greater catalytic cooperativity than SH_1-modified myosin. The catalytic cooperativity of rigor-modified myosin was also detected in measurements of myofibrillar Mg^{2+} ATPase activity (Fig. 5) that may be related to its decreased extent of shortening (Fig. 2). Yet, S-1 obtained from the myosin in rigor-labeled myofibrils showed a linear loss of actin-activated ATPase activity with the loss of K^+ (EDTA) ATPase activity (Fig. 5). Thus, the catalytic cooperativity is expressed only in the filamentous form of myosin.

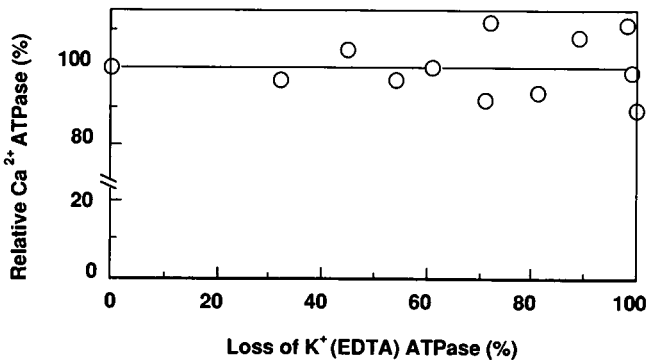

FIGURE 3 Ca^{2+} ATPase activities of IANBD-labeled myosin. Myosin was modified to varying extents with IANBD. The Ca^{2+} ATPase activities of IANBD-labeled myosin are plotted versus the loss of K^+ (EDTA) ATPase activities, which in this case measures the extent of SH_2 labeling. No change in Ca^{2+} ATPase activities are expected for specific SH_2 labeling (Reisler et al., 1974).

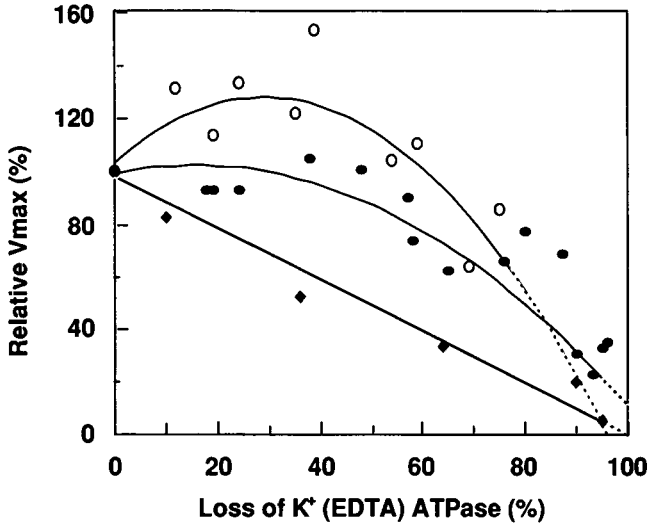

FIGURE 4 Maximum actin-activated ATP turnover rates for SH_1-, SH_2-, and rigor-modified myosins. The V_{max} plus the basal Mg^{2+} ATPase activities of SH_1- (●), SH_2- (◆), and rigor-modified (○) myosins are shown for varying degrees of modification as estimated by the loss of K^+ (EDTA) ATPase activities. Myosin was recombined (at 1.0 μM heads) with various amounts of unlabeled actin (1–60 μM) and assayed for ATPase activity in (mM) 25 KCl, 4 $MgCl_2$, 4 ATP, and 10 imidazole, pH 7.0, at 25°C. The extrapolated V_{max} for unmodified myosin was ~3.0 s^{-1}. The curves shown indicate the best polynomial fit and dashed lines indicate extrapolations.

Motility assays of modified myosins

The main observation of the motility assays was that none of the labeled myosins, SH_1, SH_2, or rigor-modified, produced detectable sliding of actin filaments. When mixed with unmodified protein, the labeled myosins inhibited the sliding of actin. The inhibition of sliding velocities of actin filaments by modified myosins could be ordered according to the ability of the modified myosins to induce catalytic cooperativity (see Fig. 6). Filamentous rigor-modified myosin inhibited sliding at very low extents of modification. Most of the sliding was lost when as little as 1% rigor-modified myosin was mixed with unmodified myosin. Similar results were obtained from myosin extracted from rigor-modified myofibrils modified to as little as 16% loss of K^+ (EDTA) ATPase activity (data not shown). Myosin modified on

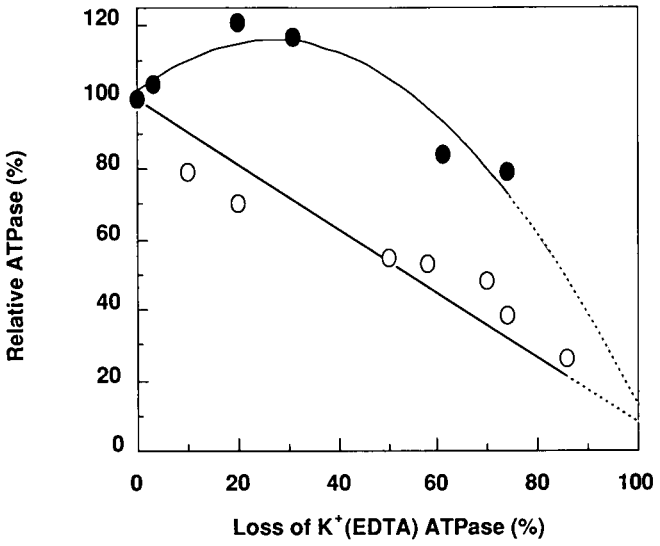

FIGURE 5 A comparison of myofibrillar and acto-S-1 turnover rates of ATP from rigor-modified myofibrils. The Mg^{2+} ATPase activities of myofibrils (●), labeled to varying extents with PM under rigor conditions, were measured in (mM) 30 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 imidazole, pH 7, and 4 ATP at 25°C in the same buffer as described in Fig. 4. S-1 (○) was extracted from these modified myofibrils, recombined (at 1.0 μM heads) with various amounts of unlabeled actin (1–60 μM), and assayed for ATPase activity in (mM) 5 KCl, 4 $MgCl_2$, 4 ATP, and 10 imidazole, pH 7.0, at 25°C. The maximum turnover rates (for unmodified proteins: S-1 = 10.0 s^{-1} and myofibrils = 1.1 s^{-1}) were normalized to control values and plotted vs. the loss of K^+ (EDTA) ATPase activities (which measures the percentage of modified heads). The curves shown indicate the best polynomial fit and dashed lines indicate extrapolations.

SH_1 inhibited sliding less than rigor-modified myosin and induced less catalytic cooperativity (Figs. 4 and 6). The SH_2-modified myosin showed no apparent catalytic cooperativity (Fig. 4) and less inhibition of sliding velocity (compared with rigor-modified myosin) until significant fractions of the myosin were inactivated by the modification. In each case, modifications inhibited both the velocity of the moving actin filaments and the proportion of actin filaments that were moving (data not shown).

The fraction of modified heads in myosin samples can be varied in two ways: samples could be modified to different extents by varying their exposure to the modifying reagent (sequentially modified) or, alternatively, myosin could be modified to near completion and then mixed with unmodified myosin to yield the desired degree of modification (mixed modified). Differences in the method chosen can influence the results obtained in motility assays (Chaen et al., 1991). Sequentially modified myosin yields slightly higher sliding velocities of actin filaments than mixed modified myosin (data not shown). This discrepancy may be due to the presence of slightly larger fraction ATP-insensitive myosin heads in mixed myosin.

Another factor that might influence the sliding of actin in the motility assay is whether myosin is applied to the coverslip as monomers or as filaments. No significant differences in sliding velocities were observed between monomeric and filamentous forms of SH_1- and SH_2-modified myosins (data not shown). However, partially rigor-modified myosin yielded higher actin sliding velocities when applied as monomers than as filaments to the coverslip (see Fig. 7). Since rigor-modified myosin is labeled in several places, as mentioned above, this effect cannot easily be attributed to a specific modification. It is possible that a combination of modifications contributes to the difference in sliding velocities of actin over monomeric and filamentous rigor-modified myosin.

Binding of myosin heads to actin

A parameter of obvious importance in attempts to correlate the different modifications of myosin with their effects on actomyosin ATPase activity and the sliding velocity of actin filaments is the binding of myosin heads to actin. Only small changes in the binding of S-1 to actin were detected upon SH_1- or rigor-modification of myosin by PM. The binding of S-1 to actin in the presence of ATP as estimated by light scattering increases by 30% for both SH_1- and rigor-modified S-1 (data not shown). This result is in good agreement with previous estimates of binding for SH_1-labeled S-1 to actin in the presence of ATP (Chalovich and Eisenberg, 1982; Root et al., 1991). The apparent binding constant, K_m, can be determined

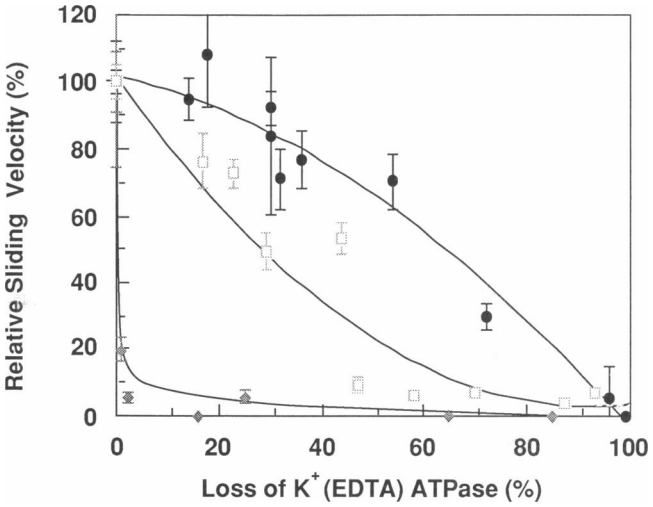

FIGURE 6 Actin sliding velocities over modified myosins. SH_1- (□), SH_2- (●), and rigor-modified (♦) myosin filaments were applied to the coverslip for in vitro motility assays of actin sliding velocity in (mM) 25 KCl, 1 EGTA, 5 MgCl2, 10 DTT, 25 imidazole, pH 7.0, at 25°C. The mean sliding velocity (error bars indicate the 99% confidence limits) was plotted vs. the K^+ (EDTA) ATPase activities (which measures the percentage of modified heads). The mean actin sliding velocities were normalized to the value for actin sliding over unmodified myosin on the day of the assays (for typical sliding velocities, see Materials and Methods). The curves shown indicate the best polynomial fit and dashed lines indicate extrapolations.

from double reciprocal plots of acto-S-1. The K_m decreases about threefold as SH_1 is modified with PM (Root et al., 1991). During rigor-modification, there is no significant change in the K_m (data not shown).

The binding of S-1 to actin is greatly decreased by ATP. If ATP is available in limited quantities, then the increased binding between myosin heads and actin will decrease the sliding velocity in motility assays and increase the actomyosin ATPase activity (Harada et al., 1990). In solution and at high concentrations, actin forms organized "sleeve" structures around myosin filaments (Ikemoto et al., 1966). If the diffusion of ATP at the actomyosin interface is limited in this protein matrix, as it is in fibers (Glyn and Sleep, 1985), then increasing the ATP concentration should decrease the binding between actin and myosin and consequently decrease the actomyosin ATPase activity. The differences between acto-S-1 and actomyosin ATPase activities were enhanced by increasing the ATP concentration from 3 to 10 mM at high actin concentration and 10 mM $MgCl_2$ (see Fig. 8). [Even greater inhibition of actomyosin ATPase activity was observed at lower ionic strength when the $MgCl_2$ concentration was kept at 2 mM as opposed to 10 mM (data not shown).] The increase in ATP concentration had no significant effect on the Mg^{2+} ATPase activity of myosin alone (data not shown).

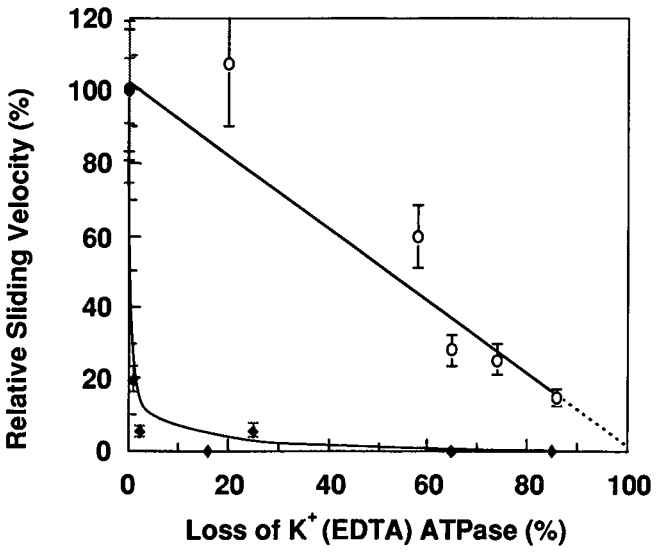

FIGURE 7 Actin sliding velocities over monomeric vs. filamentous rigor-modified myosin. Monomeric (○) or filamentous (◆), rigor-modified myosin was applied to a coverslip for in vitro motility assays. The motility assays were performed under the same conditions as those described in Fig. 6. The mean sliding velocity (error bars indicate the 99% confidence limits) was plotted vs. the K^+ (EDTA) ATPase activities (which measures the percentage of modified heads). The mean actin sliding velocities were normalized to the value for actin sliding over unmodified myosin on the day of the assays (for typical sliding velocities, see Materials and Methods). Dashed lines indicate extrapolations.

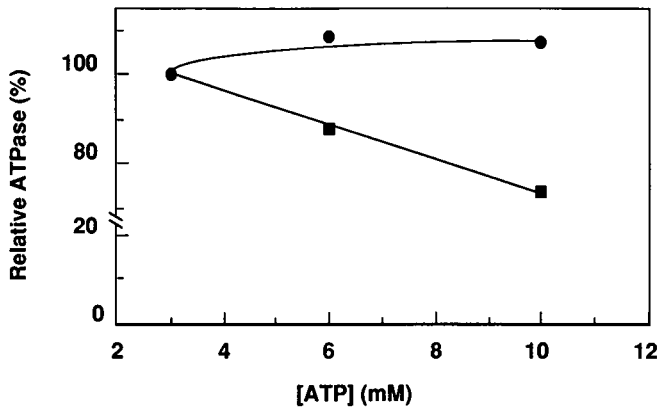

FIGURE 8 Actomyosin and acto-S-1 ATPase activities vs. ATP concentration. Myosin (■) or S-1 (●) was recombined (at 1.0 μM heads) with actin at 55 μM and assayed for ATPase activity in (mM) 25 KCl, 10 $MgCl_2$, and 10 imidazole, pH 7.0, at 25°C with 3, 6, or 10 mM ATP. The relative ATPase activities are normalized to activity values at 3 mM ATP (1.0 s^{-1} for myosin and 2.1 s^{-1} for S-1) for sake of comparison.

DISCUSSION

Effects of modification on the labeled myosin head

Chemical modifications of myosin have been used frequently to attach probes for monitoring conformational changes in the myosin head (Thomas, 1987). If the information obtained from these probes is to reflect what is happening in the intact system, the perturbation of labeled myosin's function should be minimal. Since a muscle fiber is a complex biological system, damage to a few myosin heads may not necessarily produce a large inhibition in the fiber's properties. Modified myosins must be examined in a simpler environment, in solution, to determine if the modification alters the function of the labeled head. The present study indicates that each of three different types of chemical modifications, SH_1, SH_2, and rigor-labeling of myosin, inhibits the actomyosin ATPase and actin sliding functions of myosin.

Although a rather specific modification of SH_1 has been achieved even in the muscle fiber (Duke et al., 1976), the specific modification of SH_2 was possible only on S-1 in solution until very recently (Ajtai and Burghart, 1989). Ajtai and Burghart (1989) showed that SH_2 could be labeled directly in the fiber with IANBD under the conditions that are known to enhance the reactivity of SH_2 relative to SH_1. The greater reactivity of IANBD for SH_2 than that of most other reagents could be due to its largely hydrophobic character. The present study shows that a modified version of the procedure used in fibers can be applied to labeling myosin on SH_2 in solution with a relatively high specificity. Even when myosin was modified to between 80 and 100% loss of K^+

(EDTA) ATPase activity, the loss of this activity correlated with 1.12 ± 0.09 mol of IANBD incorporated per mole of myosin head. The Ca^{2+} ATPase activities of IANBD-labeled myosin also correlated with previous characterizations of SH_2 modification on S-1 (Reisler et al., 1974), and the modification of SH_1 on IANBD-myosin with IAA brought the Ca^{2+} ATPase activity to zero. In the previous work (Ajtai and Burghart, 1989), myosin extracted from IANBD-modified fibers showed higher incorporation of IANBD per loss of K^+ (EDTA) ATPase than found here for myosin labeled in solution. The discrepancy might be accounted for by difficulties in extracting sufficient quantities of purified myosin from lightly labeled fibers for precise determination of its IANBD content or by a less specific reaction of IANBD with SH_2 groups in muscle fibers at pH 7 than in solution at pH 8.

In nearly completely modified myosin, for each type of modification examined in this study, no movement was observed by eye nor detected by the ExpertVision system. The noise in computer assisted analysis of actin filaments bound to myosin in the absence of ATP yielded a baseline velocity of 0.03 μm/s with our system (data not shown). The limit of detection of movement by eye during an observation period of 1 min is marginally more sensitive, given that translocation of an actin filament by 1 μm can be detected by eye: 1 μm/60 s = 0.02 μm/s. This analysis indicates that the modified myosins are ≥100 times slower than unmodified myosin in the motility assay. However, the possibility that modified myosin undergoes a very slow crossbridge cycle cannot be excluded (Warshaw et al., 1990).

Our results corroborate those of Harrington et al. (1975), who found that the shortening of actomyosin threads was slowed and eventually halted by copolymerization with increasingly higher fractions of SH_1-labeled myosin. Chaen et al. (1991) also reported similar results with SH_1-labeled S-1 and HMM in the motility assay. We cannot explain the results of Srivastava and Wikman-Coffelt (1980) that *N*-ethylmaleimide-labeled myosin had no effect on the force or velocity of similar actomyosin threads. Crowder and Cooke (1984) reported that in relaxed-modified muscle fibers, the isometric force and V_{max} of shortening were unaltered by thiol modification until more than half of the K^+ (EDTA) ATPase activity of extracted myosin was lost, and then both isometric force and V_{max} of shortening were rapidly lost with additional loss of K^+ (EDTA) ATPase activity. It is only possible to speculate on the differences between the results in the fiber and those in the motility assay. One possibility is that SH_1-labeled myosin cannot compete well with unmodified myosin for binding to actin under fiber conditions (organized protein matrix and physiological salt conditions). Since most estimates of the percentage of myosin heads engaging in crossbridge interaction at any given time in the isometric fiber are in the range 20–70% (Duong and Reisler, 1989; see Cooke, 1986, for a review) and assuming the lower end of this range, it is quite possible that the unmodified heads could compensate for the modified heads. Another possibility is that the mechanisms for generating unloaded sliding and isometric force are separate as suggested by some recent models (Oplatka, 1990; Harrington et al., 1990). In this case, the modified heads might be capable of contributing to isometric force, since virtually no work is being done under isometric conditions that might require high rates of ATPase activity. The fact that isometric fibers have lower ATPase activity than isotonically contracting fibers is consistent with such a model (Kushmerick and Davies, 1969; Houadjeto et al., 1991).

"Activation-interference model"

Each modification examined, SH_1, SH_2, and rigor-modified myosin, inhibited sliding velocity and actomyosin ATPase activity (Figs. 4 and 6). The profiles of inhibition of actomyosin ATPase activity and sliding velocity were very different for each modification. The SH_1- and rigor-modified myosins exhibited catalytic cooperativity in their actomyosin ATPase activities (Fig. 4). Catalytic cooperativity for SH_1-modified myosin was described in our previous study (Root et al., 1991) and appears to result from the elevation of the actin-activated ATPase activity of unmodified heads induced by modified myosin heads in the same myosin filament. Although numerous possible models could explain this phenomenon (Root et al., 1991), the data of this study suggest that the mechanism by which modified heads elevate the actomyosin ATPase activity of unmodified heads is related to the degree to which the modified heads inhibit sliding of partially modified filaments.

The concept that slower sliding filaments have higher actomyosin ATPase activity is supported by a number of observations. Hill (1964) first noted that the energy used by muscle fibers increased when an external load was applied to the fibers to slow their rate of shortening. Explanations of this observation included the possibility that a myosin head might need to interact with multiple sites on the actin molecule before ATPase activity would be accelerated (Huxley, 1973) and that a weakly bound myosin head could be torn from the actin filament in a rapidly shortening fiber before it could productively interact with actin (Homsher, 1987). In a related observation, the actomyosin ATPase activity in the motility chamber increased at ATP concentrations low enough to inhibit the observed sliding velocity (Harada et al., 1990). Along similar lines of reasoning, the proposed model could also explain the difference in actin-activated ATPase activities between filamentous myosin and its proteolytic fragments that cannot form filaments (Silverman et al., 1972; Root et al., 1991).

The "activation-interference" model would predict that the apparent interaction distance per ATP hydro-

lyzed would be reduced if an external load were applied to slow sliding velocity, since the actin-activated ATPase activity would increase with slower sliding velocities. The dependency of the myosin head interaction distance per ATP on external loads is consistent with this model (Harada et al., 1990; Higuchi and Goldman, 1991). The concept that the amount of ATP hydrolyzed does not necessarily correlate with a discrete interaction distance also has been illustrated by the use of modified actins (Prochniewicz and Yanagida, 1990; Schwyter et al., 1990).

The above model also has implications for measurements of actomyosin ATPase activity. Pope et al. (1981) first reported the biphasic nature of double reciprocal plots of actin-activated myosin ATPase activity. They suggested that it might be related to the formation of "sleeve" structures of actin around myosin filaments observed at high actin concentrations (Ikemoto et al., 1966; Nonomura and Ebashi, 1974). Our results suggest how this sleeve formation might affect the rate of ATPase activity. Fig. 8 illustrates that as the ATP concentration is increased in the millimolar range, the actomyosin ATPase activity decreases. These data are consistent with the possibility that the formation of sleeves limits the diffusion of ATP at the actomyosin interface. The diffusion of ATP is limited in muscle fibers (Glyn and Sleep, 1985), which is the organized system that sleeve formation mimics (Ikemoto et al., 1966; Nonomura and Ebashi, 1974). If insufficient ATP is available for the myosin, its affinity for actin will increase, thereby slowing the sliding velocity and increasing the ATPase activity (Hill, 1964; Harada et al., 1990). Interestingly, SH_1-modification, which inhibits sliding velocity, inhibits the biphasic character of actin-activated myosin ATPase activity as well (Root et al., 1991).

Mechanism of actin sliding inhibition

The mechanisms by which SH_1- and rigor-modified myosins inhibit the sliding velocity of unmodified cross-bridges are not clear. Myosin modified with *p*-nitrophenylanedimaleimide inhibited the sliding velocity of unlabeled myosin to a similar degree to the inhibition by SH_1-labeled myosin observed in the present study (Warshaw et al., 1990). Warshaw et al. (1990) presented a model whereby the modified heads or slowly cycling heads could place an internal load on the sliding actin filament when they are pulled into a negatively strained orientation. Tawada and Sekimoto (1991) have proposed that the inhibition may be due to a frictional drag produced by nonproductive interactions of modified myosin with actin. Another possible model is that this modification decreases the off rate of myosin fragments in solution. A decreased off rate for SH_1-labeled HMM may be deduced from observations on its kinetics (Mulhern and Eisenberg, 1976). If the off rate for myosin interaction with actin in the motility assay is reduced, the sliding velocity could be inhibited, because the modified head will tightly hold on to actin during another myosin's powerstroke (Warshaw et al., 1990; Tawada and Sekimoto, 1991).

Modification of myofibrils in rigor buffer inhibits the extent of their shortening at much lower losses of K^+ (EDTA) ATPase activity than a modification under relaxed conditions (Fig. 2). Similarly, sliding velocity in the motility assay is also inhibited when small fractions of rigor-modified myosin are copolymerized with unmodified myosin, whereas considerably less inhibition is observed with SH_1-labeled myosin (Fig. 6). In addition, the catalytic cooperativity is greater with rigor-modified than with SH_1-labeled myosin (Fig. 4). One of several possible explanations of these results is that the rigor-modified myosin has a competitive advantage in binding to actin over the unmodified myosin with which it is copolymerized. The possibility that this competitive advantage is related to myosin filament structure is supported by two findings. First, less inhibition of sliding velocities occurred when rigor-modified myosin was applied as monomers as opposed to filaments (Fig. 7). Second, S-1 made from the rigor-modified myosin had a binding constant similar to SH_1-labeled myosin (data not shown). One way to conceptualize the differences between SH_1- and rigor-modified myosins is that the rigor-modified heads might extend farther out from the thick filament than unmodified or SH_1-labeled myosins, thus giving the rigor-modified myosin greater opportunity for interaction with actin filaments. Another way of explaining these results is that the rigor modifications introduce new contact sites between myosin molecules in the myosin filament that in turn inhibits the rotational flexibility of the head and the detachment rate of actin from myosin. Unfortunately, the modification or combination of modifications responsible for the effects observed in rigor-modified myosin filaments cannot be precisely determined, since ≥ 10 thiols are modified to some extent on a double-headed myosin molecule.

The correlation between inhibition of sliding velocities in the motility assay and elevation of actomyosin ATPase activities measured in solution is only qualitative (Figs. 4 and 6). Quantitative discrepancies might arise because actin in the sliding filament assay is quite sensitive to any impediments to its motion (Umemoto and Sellers, 1990). In a study of Warshaw et al. (1990), $\sim 1\%$ of overmodified S-1 was sufficient to completely stop all sliding of actin. If an actin filament ever encounters a region that does not support its sliding, it will be trapped there. In solution, actin and myosin that are bound together can still interact with many other actin and myosin filaments.

In summary, the labeling of SH_1 or SH_2 groups on myosin as well as rigor modification of this protein in myofibrils inhibit both the actomyosin ATPase activity and the sliding velocity of actin in the motility assay. Labeled myosin heads that impede the sliding velocity of

actin over unmodified myosin heads induce catalytic cooperativity in the actin-activated ATPase activity of myosin filaments. The catalytic cooperativity is proposed to result from the inhibition of actin filament sliding over myosin. S-1 prepared from the labeled myosin does not exhibit catalytic cooperativity in actin-activated ATPase measurements. The results presented here raise questions regarding the nature of the information obtained in activated muscle fibers from probes attached to the reactive thiols on the myosin heavy chain.

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