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Catalytic Cooperativity Induced by SH_1 Labeling of Myosin Filaments†

Douglas D. Root,* Pearl Cheung, and Emil Reisler
Molecular Biology Institute and Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024

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ABSTRACT: Modifications of SH_1 groups on isolated myosin subfragment 1 (S-1) and myosin in muscle fibers affect differently the acto-S-1 ATPase and the fiber properties. Consistent with the findings of earlier work on fibers, the modification of SH_1 groups in relaxed myofibrils with phenylmaleimide caused a loss of their shortening. This loss paralleled the decrease in the V_{max} of extracted myosin but was not linear with the extent of SH_1 labeling. Strikingly, the decrease in V_{max} of S-1 prepared from the modified myofibrils was directly proportional to the extent of SH_1 labeling. The specificity of SH_1 labeling in myofibrils was verified by ATPase activities, thiol titrations, radiolabeling experiments, and comparisons to myosin labeled on SH_1 in solution. To test for intermolecular interactions in the myosin filaments and their contribution to the differences between S-1 and myosin, the catalytic properties of copolymers of myosin were examined. Copolymers of myosin and rod minifilaments were formed in 5 mM citrate-Tris (pH 8.0) buffer, and their homogeneity was verified by sedimentation velocity analysis. The inhibition of actomyosin ATPase by rod particles was related to the decrease in the K_m value. When rod particles were replaced in these minifilaments by SH_1-modified myosin, the ATPase of the copolymers was increased over that of the combined ATPases of the individual filaments. The actomyosin ATP turnover rates on the unmodified heads were increased severalfold by the modified heads. These results demonstrated that the catalytic function of myosin heads in the filaments is affected by the interactions of vicinal myosin molecules with actin. The results also suggest that SH_1 modification of myosin may impede its ability to undergo a normal crossbridge cycle.

Muscles produce force by cyclic crossbridge interactions between actin and myosin which are coupled to the hydrolysis of ATP. Understanding these processes depends on the coordination of studies done on muscle fibers and the work on solubilized muscle proteins and protein complexes. Mechanisms of ATP hydrolysis have been investigated extensively in solution (Sleep et al., 1981), and measurements in fibers and myofibrils are consistent with these mechanisms (Sleep, 1981; Glyn & Sleep, 1985). Foundations for the understanding of actomyosin interaction in fibers were laid by detailed solution investigations on actin and myosin [for a review, see Cooke (1986)]. Although the elucidation of the possible changes in crossbridge structure and orientation during contraction depends on the analysis of fiber experiments, these studies are linked to solution work as well. Understanding the effects of introducing probes to monitor changes in crossbridges must rely on characterization in solution of the chemical reaction and the labeled proteins it generates.

The most popular probe site on myosin has been the reactive SH_1 group on the myosin head. The effects of modifications of sulfhydryl groups on myosin subfragment 1 (S-1)[1] are well characterized (Sekine & Kielley, 1964; Silverman et al., 1972; Mulhern et al., 1975; Mulhern & Eisenberg, 1976, 1978). When the most reactive thiol, SH_1, is specifically labeled, the K^+ (EDTA) ATPase and actin-activated ATPase are abolished, while the basal Mg^{2+} and Ca^{2+} ATPases are elevated (Sekine & Kielley, 1964; Mulhern & Eisenberg, 1976). The inhibition of acto-S-1 ATPase activity by labeling SH_1 is

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* Address correspondence to this author at the Department of Chemistry and Biochemistry, University of California, Los Angeles.

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HMM, heavy meromyosin; kDa, kilodalton(s); NEM, *N*-ethylmaleimide; OD, optical density; pPDM, *p*-phenylenedimaleimide; PM, phenylmaleimide; S-1, myosin subfragment 1; SDS, sodium dodecyl sulfate.

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caused by decreases in the V_{max} and the apparent K_m. Extensive modification of SH_1 on myosin was similarly found to result in large losses of actomyosin ATPase activity (Mulhern & Eisenberg, 1978). When actomyosin threads were made from myosin labeled to varying extents, their ability to shorten was lost though not in direct proportion to the amount of labeling (Harrington et al., 1975). These results suggest that myosin heads modified on SH_1 do not go through the crossbridge cycle or generate force in the same way as unmodified heads.

A different conclusion was reached on the basis of SH_1 modifications in muscle fibers. The loss of isometric tension and maximum velocity of contraction as a function of SH_1 modification in fibers has been studied in detail by Crowder and Cooke (1984). In that work, the loss of force did not correlate with the SH_1 labeling of either one or both of the myosin heads. Consequently, Crowder and Cooke (1984) concluded that SH_1 groups could be labeled with relative impunity in the fiber, and modifications elsewhere on myosin or in the fiber were responsible for the loss of contraction. These findings have been used to validate spectroscopic studies on crossbridges in which the probe was attached to SH_1 groups (Mendelson et al., 1975; Borejdo & Putnam, 1977; Thomas & Cooke, 1980; Thomas et al., 1980, 1983, 1988; Cooke, 1981; Burghardt et al., 1984, Barnett & Thomas, 1987; Belágyi & Röth, 1987; Thomas, 1987; Ludescher & Thomas, 1988; and others).

Because the interpretation of spectroscopic crossbridge studies relies on the assumption that SH_1 labeling does not alter the normal crossbridge cycle, it is important to resolve the apparent discrepancy between the functional consequences of SH_1 modification on myosin in the fiber and on S-1 in solution. In this study, we examine the possibility that this discrepancy between solution and fiber work is caused by cooperative interactions between myosin heads in the same myosin filament. By alteration of the composition of myosin filaments, their affinity for actin and actomyosin ATPase activities are changed accordingly. This cooperative effect, which is seen in solution, is correlated with the changes in the properties of fibers upon modification. Models for how this cooperative effect works at the molecular level in the fiber are also discussed.

Materials and Methods

Reagents. Trypsin, soybean trypsin inhibitor, TLCK-treated α-chymotrypsin, phenylmethanesulfonyl fluoride, papain, Bis-Tris, *N*-ethylmaleimide (NEM), ATP, DTNB, EGTA, and β-mercaptoethanol were procured from Sigma Chemical Co. (St. Louis, MO). 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 *N*-[2,4,6-^{3}H]phenylmaleimide were obtained from Eastman Kodak (Rochester, NY) and Amersham International (Buckinghamshire, England), respectively. PPO and EDTA were procured from Fisher (Fairlawn, NJ). The Bradford protein assay was purchased from Bio-Rad (Richmond, CA). Millipore-filtered distilled water and analytical-grade reagents were used in all experiments.

Proteins. Myofibrils were prepared from rabbit skeletal muscle as previously described (Duong & Reisler, 1989). Myosin was extracted from these myofibrils in a solution of 300 mM KCl, 10 mM ATP, 10 mM $MgCl_2$, 2 mM sodium pyrophosphate, and 10 mM imidazole, pH 7.0, and then centrifuged for 2 h at 4 °C in a Beckman Ti 50 rotor at 35000 rpm. S-1 was prepared from the myofibrils by digesting 10 mg/mL myofibrils in 120 mM KCl, 2 mM EDTA, and 10 mM Tris, pH 8.0, with 0.2 mg/mL α-chymotrypsin for 45 min at 22 °C (Duong & Reisler, 1989). The cleaved myofibrils were extracted first as for myosin above; then the supernatant was dialyzed against 40 mM KCl and 10 mM imidazole, pH 7.0, and centrifuged for 2 h at 4 °C in a Beckman Ti 50 rotor at 35000 rpm. The concentration of the myofibrils was estimated by spectrophotometric readings in 5% (w/v) SDS using $E^{1\%}_{280nm}$ = 7.0 cm^{-1} (Sutoh & Harrington, 1977).

Purified rabbit skeletal myosin was prepared as described (Godfrey & Harrington, 1970). S-1 was obtained from myosin with α-chymotrypsin and separated according to Weeds and Pope (1977). Myosin rod was prepared by the method of Oriol-Audit et al. (1981). The concentrations of myosin, S-1, and rod were measured spectrophotometrically by using $E^{1\%}_{280nm}$ = 5.5 cm^{-1} in high salt for myosin (Godfrey & Harrington, 1970), $E^{1\%}_{280nm}$ = 7.5 cm^{-1} for S-1 (Wagner & Weeds, 1977), and $E^{1\%}_{280nm}$ = 2.0 cm^{-1} for myosin rod (Harrington & Himmelfarb, 1972). Rabbit skeletal muscle actin was extracted from acetone powder (Spudich & Watt, 1971). Its concentration was determined spectrophotometrically by using $E^{1\%}_{280nm}$ = 11.0 cm^{-1} (West et al., 1967).

Labeling Myosin's Head. Specific labeling of SH_1 groups on the myosin head has been described both in solution (Burke & Reisler, 1977) and in myofibrils (Duke et al., 1976; Thomas et al., 1980). Myofibrils (2 mg/mL) were modified under relaxing conditions in the presence of 40 mM KCl, 2 mM EGTA, 2 mM $MgCl_2$, 2 mM sodium pyrophosphate, and 10 mM imidazole, pH 7.0, with concentrations of PM up to 0.15 mM (dissolved initially in dimethylformamide so the final amount of organic solvent was less than 3%) for 30 min at 4 °C. Isolated myosin at 7 mg/mL was labeled with pNPM (2-fold molar excess) in 0.6 M KCl and 10 mM imidazole, pH 7.0, for 15 min at 4 °C. The modification of myosin (7 mg/mL) with a 4-fold molar excess of NEM (dissolved in ethanol) was performed in 0.6 M NaCl and 10 mM Bis-Tris, pH 7.0, at 4 °C for 1 h. All reactions were terminated with a 10-fold molar excess of DTT or β-mercaptoethanol over the modifying reagent. The extent of SH_1 modification was checked by Ca^{2+} and K^+ (EDTA) ATPase activities. The extent of SH_1 modification of both myosin and S-1 extracted from modified myofibrils was identical as judged from K^+ (EDTA) ATPase activities (data not shown). Concentrations of the modified proteins were determined by Bradford protein assays (Bradford, 1976), as modified by Bio-Rad (Richmond, CA), according to the manufacturer's instructions.

Turbidometric Assays of Shortening. Myofibrils were suspended in 30 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM imidazole, pH 7.0, and filtered through one layer of cheesecloth. The turbidity at 650 nm was monitored by continuously pumping a 0.1 mg/mL suspension of myofibrils through the sipper cell of a Beckman Instruments (Fullerton, CA) Model 25 spectrophotometer. Addition of 20 μM ATP initiated the myofibrilar contraction. Higher concentrations of ATP did not increase the response. The increases in the OD were measured and were approximately 0.1 OD unit for unmodified myofibrils. Changes in turbidity were observed within a second, and corresponded to microscopic observations of the myofibrils. Characterization of the turbidity assay of myofibrilar shortening and documentation of its reversibility have been done previously (Maruyama & Kominz, 1969; Hotta & Bowen, 1970; 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 30 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM imidazole, pH 7.0, and 4 mM ATP at 25 °C. Actin-activated ATPases for S-1 and myosin filaments were measured at 25 °C in 2 mM $MgCl_2$, 10 mM imidazole, pH 7.0, 4 mM ATP, and at KCl concentrations as specified in the figure legends. Myosin minifilaments were prepared by the method of Reisler et al. (1980) with a final dialysis solution of 5 mM citrate–Tris, pH 8.0, at 4 °C. Copolymers of myosin and either rod or labeled myosin were made by mixing appropriate ratios of their minifilaments and allowing them to exchange at 25 °C for 30 min in 5 mM citrate–Tris, pH 8.0. The actin-activated ATPase activities of minifilaments were measured in 5 mM citrate–Tris, pH 8.0, at 25 °C with salt concentrations as described in the figure legends. Determinations of the amount of inorganic phosphate released during the assays were made as previously described (Reisler, 1980).

Analytical Ultracentrifugation. The sedimentation experiments were carried out at 20 °C in a Spinco Model E analytical ultracentrifuge equipped with a UV scanner. Sedimentation was monitored at 280 nm at a velocity of 28 000 rpm which does not influence the polymerization of the minifilaments (Reisler et al., 1980).

Binding Assay. Relative binding affinities of NEM, PM, and unmodified S-1 for actin were determined by 90° light scattering at 325 nm in a Spex Fluorolog spectrophotometer (Spex Industries, Inc., Edison, NJ) at 25 °C. Actin (3 μM) was added to between 5 and 15.6 μM S-1 in 20 mM imidazole, pH 7.0, and 2 mM $MgCl_2$ and allowed to polymerize. Next, 2 mM ATP was added, causing a reduction in scattering until the ATP was hydrolyzed. Light scattering due to free actin and S-1 was measured separately and subtracted out. Labeling by NEM and PM was 83% and greater than 95%, respectively, as measured by the loss of their K^+ (EDTA) ATPase activities. The light scattering varied linearly with the amount of S-1 added ($r^2 \geq 0.92$). On the basis of light-scattering measurements of the fraction of actin bound to unmodified S-1 in the presence of ATP, a K_a of 6×10^3 M^{-1} was estimated. This value of K_a agrees with values between 2×10^3 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 & 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; then 0.2 mL of the titration reagent (1 mM DTNB, 0.1 M KCl, and 10 mM $K_2HPO_4 \cdot 3H_2O$) was added, incubated for 15 min at 22 °C, and read by using $E_{412nm} = 1.36 \times 10^4$ M^{-1} (Ellman, 1959).

Fluorography. Myofibrils were labeled with *N*-[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 partially papain-cleaved S-1 (Applegate & Reisler, 1983), were run on a 5–20% (w/w) polyacrylamide linear gradient gel made according to the method of Laemmli (1970). Fluorography was performed with PPO on Kodak X-Omat AR film as described (Bonner, 1984). A Biomed Instruments (Fullerton, CA) Model SL-504-XL soft laser densitometer equipped with an integrator and interfaced to an Apple IIe computer was used to quantify the bands on the fluorogram.

Results

Strategy for Experiments. The hypothesis tested in this work was that cooperative effects in the myosin filaments are responsible for the apparent differences in the functional consequences of SH_1 modification on S-1 and in muscle fibers. To test this hypothesis, two groups of experiments involving SH_1 modification in myofibrils and SH_1 labeling on isolated S-1 and myosin were carried out. Myofibrils were used rather than fibers because of the ease of labeling proteins in this system, and because adequate quantities of myosin could be extracted for subsequent solution work. The first aim of myofibrilar modifications was to verify the specificity of SH_1 labeling in that system. The second and main goal of these experiments was to determine whether the loss of myofibrilar shortening is correlated with the extent of SH_1 modification. This correlation suggested that the nonlinear dependence of myofibrilar contractility and Mg^{2+} ATPase on SH_1 labeling may stem from cooperative effects in the myosin filaments. To test this explanation (that the properties of partially labeled myofibrils and fibers are affected by the catalytic nonhomogeneity of myosin filaments), actin-activated ATPases of copolymers of labeled and unlabeled myosin were measured.

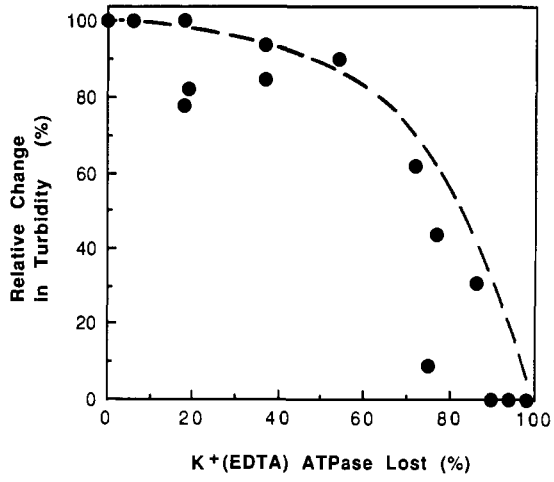

FIGURE 1: Loss of contractility upon modification of myofibrils with PM. Myofibrils (●), modified to varying extents under relaxing conditions with PM, were assayed for their ability to contract by using a turbidometric assay as described under Materials and Methods. Myofibrils (0.1 mg/mL) were suspended in 30 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM imidazole, pH 7, to which 20 μM ATP was added to initiate the contraction at 22 °C. The relative changes in OD measured at 650 nm are plotted on the vertical axis. The loss of K^+ (EDTA) ATPase activities in the extracted myosin, which monitors the extent of SH_1 modification, is plotted on the horizontal axis. The dashed curve is taken from isometric tension and maximum velocity of shortening measurements made by Crowder and Cooke (1984) on fibers modified with maleimide spin-label.

Copolymers of rod and myosin were used in this group of experiments to examine the effect of changes in the spacing of myosin heads on the filament on its affinity for actin. Finally, ATPase measurements on the copolymers of SH_1-modified and unmodified myosin probed the effect of labeled heads on the activity of unlabeled myosin heads.

SH_1 Modification of Myofibrils. (A) Loss of Contractility and ATPase Activity. According to earlier work, the initial reduction of force and velocity of shortening in muscle fibers upon sulfhydryl modification is much slower than the loss of K^+ (EDTA) ATPase activity of the modified myosin (Crowder & Cooke, 1984). Figure 1 shows that identical results are obtained when modifying myofibrils with phenylmaleimide under relaxed conditions. In this case, the loss of contractility was measured by monitoring the rapid changes in turbidity accompanying the contraction of myofibrils (Maruyama & Kominz, 1969; Hotta & Bowen, 1970; Kominz, 1970). The relative turbidity changes of modified myofibrils were then plotted in Figure 1 versus the relative K^+ (EDTA) ATPase

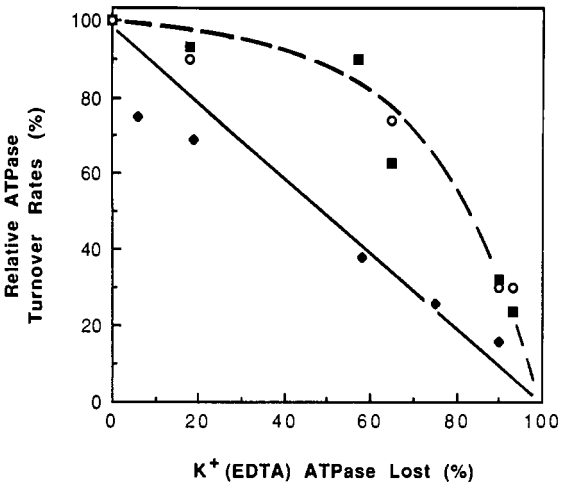

FIGURE 2: Comparisons of myofibrilar, actomyosin, and acto–S-1 turnover rates of ATP with the loss of contractility in SH_1-modified myofibrils. The Mg^{2+} ATPase activities of myofibrils (○), labeled to varying extents with PM under relaxing conditions, were measured in the same buffer as described in the legend to Figure 1 except that 4 mM ATP was added to initiate the reaction at 25 °C. Myosin (■) was extracted from these modified myofibrils, recombined (at 1 μM heads) with various amounts of unlabeled actin (1–60 μM), and assayed for ATPase activity in 25 mM KCl, 4 mM $MgCl_2$, 4 mM ATP, and 10 mM imidazole, pH 7.0, at 25 °C. S-1 (♦) was also obtained from modified myofibrils, recombined (at 1 μM heads) with various amounts of unlabeled actin (1–60 μM), and assayed for ATPase activities in 5 mM KCl, 4 mM $MgCl_2$, 4 mM ATP, and 10 mM imidazole, pH 7.0, at 25 °C. The maximum turnover rates (for unmodified proteins: S-1 = 6.8 s^{-1}, myosin = 3.1 s^{-1}, and myofibrils = 1.3 s^{-1}) obtained from Figure 3 and similar double-reciprocal plots were normalized to control values and plotted versus the loss of K^+ (EDTA) ATPase activities (which measures the extent of SH_1 labeling). The dashed curve represents the loss of contractility and is taken from Figure 1. The solid line represents the expected loss of force due to modification of myosin on SH_1 and in the absence of cooperative interactions. The basal Mg^{2+} ATPase plus V_{max} of acto–S-1 extrapolated to complete modification of SH_1 is about 5% of the unmodified level.

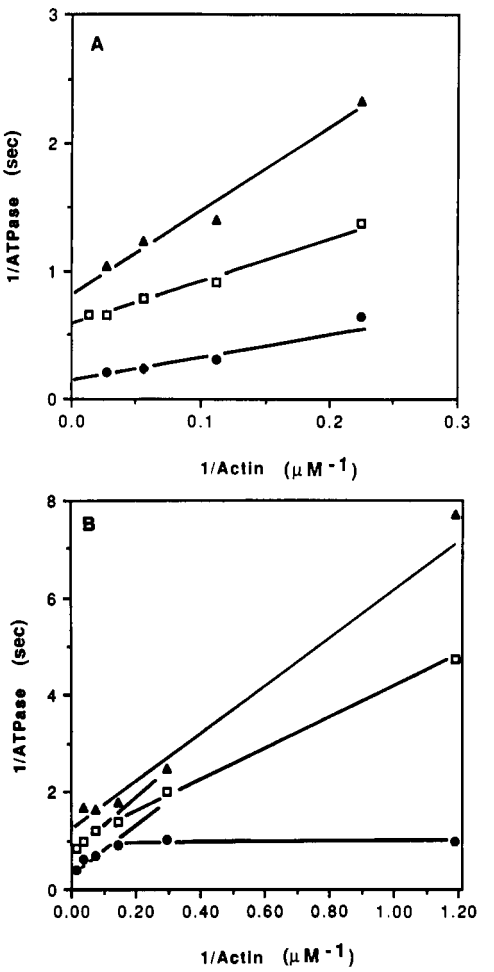

FIGURE 3: Representative double-reciprocal plots of actin-activated ATPase activities of (A) S-1 and (B) myosin extracted from modified and intact myofibrils. Myofibrils were modified with PM under relaxing conditions to varying extents as measured by the loss of K^+ (EDTA) ATPase activity of the extracted myosin. The respective high actin concentration V_{max} and K_m values for each plot were 6.8 s^{-1} and 20 μM for unmodified S-1 (●), 1.8 s^{-1} and 7 μM for 63% modified S-1 (□), 1.1 s^{-1} and 7 μM for 90% modified S-1 (▲), 3.1 s^{-1} and 14 μM for unmodified myosin (●), 2.0 s^{-1} and 8 μM for 75% modified myosin (□), and 1.0 s^{-1} and 5 μM for 90% modified myosin (▲). The conditions for the ATPase assays were described in the legend to Figure 2.

activities of the modified myosin. Since the modification is specific for SH_1, as shown below, the loss of K^+ (EDTA) ATPase activity is directly proportional to the extent of their labeling (Bailin & Barany, 1972). Thus, the loss of shortening showed an initial lag with respect to the extent of SH_1 labeling, but total loss of contraction occurred as complete modification of SH_1 was approached (Figure 1). The possibility that this profile might be explained by cooperative effects within the myofibril rather than nonspecific modifications (Crowder & Cooke, 1984) was examined in the following experiments.

The loss of Mg^{2+} ATPase activity of myofibrils coincided with the loss of their shortening (Figures 1 and 2). To determine whether or not modifications on the myosin molecule itself were responsible for the loss of Mg^{2+} ATPase activity, myosin was extracted from the labeled myofibrils and recombined with purified actin. Figure 2 shows that the maximum turnover rates of ATP of such reconstituted actomyosin were directly proportional to the Mg^{2+} ATPase activities obtained in myofibrils. Myosin modified to 96% loss of EDTA ATPase activity with PM in solution and mixed with varying amounts of unmodified myosin yielded similar profiles of loss of V_{max} as higher percentages of modified myosin were used (data not shown). Therefore, the nonlinear loss of V_{max} with the extent of SH_1 labeling could not be due to modifications other than on SH_1 in myosin. Yet when acto–S-1 ATPase activities of S-1 extracted from the partially labeled myofibrils were measured under similar conditions, the loss of activity was directly proportional to the extent of SH_1 modification (Figure 2). Similarly, the linear loss of acto–HMM ATPase with the modification of isolated HMM was reported in an earlier study (Reisler, 1980). Since S-1 and heavy meromyosin do not assemble as does myosin, the discrepancies in their actin-activated ATPase activities are indicative of cooperativity at the myosin filament level.

The maximum turnover rates shown in Figure 2 were derived from double-reciprocal plots of actomyosin ATPases of S-1 and myosin extracted from modified myofibrils. Representative plots for proteins obtained from myofibrils labeled to three different extents are shown in Figure 3. The double-reciprocal plots were characteristically linear for S-1 (Figure 3A; Mulhern & Eisenberg, 1976) and biphasic for the control myosin (Figure 3B; Pope et al., 1981). Modified S-1 had a lower apparent K_m for actin than the control (Figure 3A), as is characteristic of SH_1 modification (Mulhern & Eisenberg, 1978). This increased affinity for actin was also found with the labeled myosin filaments at high concentrations of actin (Figure 3B). Notably, as the myosin filament was modified, its actin-activated ATPase activities appeared to lose their biphasic kinetic profile (Figure 3B). It could be that a wider range of actin concentrations than employed here is

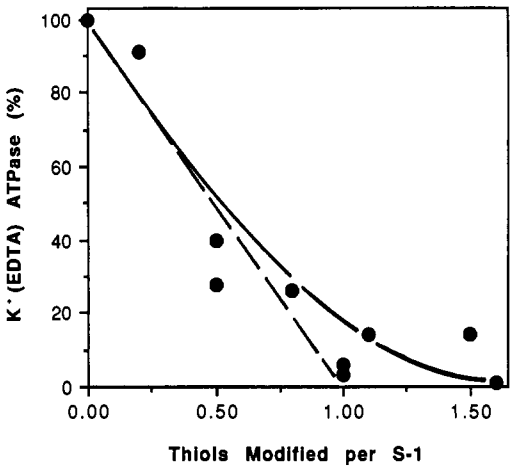

FIGURE 4: Thiol titrations of S-1 isolated from SH_1-labeled myofibrils. The number of modified thiols was determined from DTNB titrations. The K^+ (EDTA) ATPase activity was plotted versus the number of labeled thiols (●). The dashed line represents the results expected for a specific labeling of the SH_1 group on S-1.

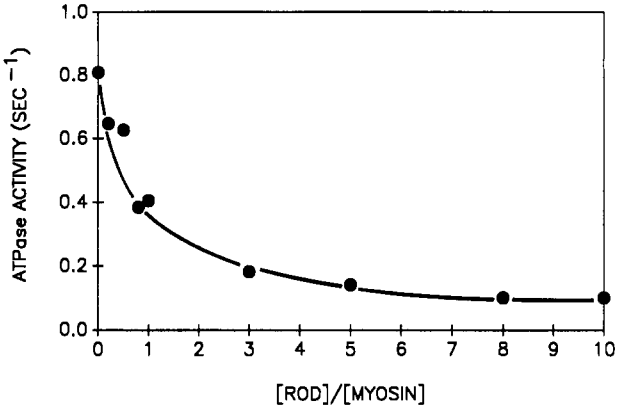

FIGURE 5: Inhibition of actin-activated ATPase of myosin minifilaments by myosin rod. Myosin and myosin rod minifilaments, prepared separately in 5 mM citrate–Tris, pH 8.0, were mixed at the indicated molar ratios and allowed to exchange for 30 min at 25 °C. The actomyosin ATPase was assayed in 5 mM citrate–Tris, pH 8.0, 2 mM $MgCl_2$, 2 mM ATP, 3 μM actin, 0.5 μM myosin, and 0–5 μM myosin rod at 25 °C. The actomyosin ATPase activity per myosin head (●) was plotted versus the molar ratio of myosin rod to myosin.

required to detect the biphasic transitions in modified myosin filaments. The apparent increased binding of the modified myosin for actin suggested a possible mechanism for the cooperative effect at the filament level of myosin: Tighter binding of partially labeled myosin filaments to actin may increase the turnover rates of the unmodified heads.

(B) Specificity of SH_1 Modification. On the basis of a linear plot of Ca^{2+} versus K^+ (EDTA) ATPases of the extracted myosin and S-1 (r^2 = 0.96, data not shown), the modification of SH_1 in the myofibril appeared to be specific (Crowder & Cooke, 1984). For both proteins, the Ca^{2+} ATPase activities increased up to 4-fold with the decrease in K^+ (EDTA) ATPase activities (data not shown). In addition to that, the number of modified thiols on S-1 extracted from the labeled myofibrils was determined by direct titrations. Figure 4 shows that the modification of only one thiol is responsible for much (at least 80 or 90%) of the loss of K^+ (EDTA) ATPase activity by S-1. A high specificity of SH_1 labeling on the myosin head is therefore indicated.

The specificity of the modification reaction of myofibrils with *N*-[2,4,6-^{3}H]phenylmaleimide was also investigated by fluorography. The relative densities of radiolabeled protein bands in the undigested myofibrils indicated that most of the labeling was in actin and myosin (data not shown). The chymotryptically digested myosin showed mostly modification of S-1 (75% of the label on myosin even when modified past complete loss of contractility) and less modification of the rod (25%). When modified to 50% loss of EDTA ATPase activity, myosin labeled in myofibrils had the following distribution of PM: 70% on the heavy chain, 20% on the rod, and 10% on the light chains. Tryptic and papain digestions of S-1 demonstrated that the label was localized to the 20-kDA tryptic fragment of the S-1 heavy chain (data not shown). These results are consistent with relatively specific modification of SH_1 in myofibrils.

The labeling of SH_1 in isolated myosin has been shown to be highly specific (Sekine & Kielley, 1964; Burke & Reisler, 1977). Thus, in a functional test of SH_1 labeling in myofibrils, we compared the kinetic consequences of thiol modification in isolated S-1 and its parent myosin with the activities of proteins recovered from labeled myofibrils. When purified myosin was labeled to 46% loss of K^+ (EDTA) ATPase in solution, both it and S-1 prepared directly from it showed the same actin-activated ATPase properties as myosin and S-1 extracted from labeled myofibrils (data not shown). The actomyosin ATPase activity was not significantly impaired by SH_1 labeling, but the acto–S-1 ATPase was decreased proportionally to the extent of SH_1 labeling (data not shown). Thus, myosin modified in the myofibril behaves similarly to myosin modified in solution.

Cooperativity of Myosin Minifilaments in Solution. (A) ATPase Activities of Myosin and Rod Copolymers. In order to account for the observed cooperative changes in actomyosin ATPase with SH_1 modification (Figures 2, 3, and 5), we have assumed the interactions of myosin heads in the filament with actin are impacted by vicinal myosin molecules. To test this hypothesis, conditions were chosen that would optimize the putative cooperative effects. Actin concentrations were chosen that gave less than 10% of the V_{max} actin-activated ATPase activity. Also, myosin minifilaments, rather than regular filaments, were chosen, since their greater solubility and smaller size (Reisler et al., 1980) allow more accurate determination of the ATPase activity (Cheung & Reisler, 1983). These measurements revealed not only that binding of minifilaments to actin could be increased by modifying some SH_1 groups with NEM but also that the affinity for actin per minifilament could be decreased by interspacing myosin molecules with rod particles.

Copolymers of myosin and rod have fewer myosin heads per minifilament and, hence, lower affinity for actin per minifilament. Figure 5 shows that as the ratio of rod to myosin is increased, the actomyosin ATPase activity per head decreases rapidly to about one-eighth of the control value. The IC_{50} (inhibitory concentration for 50% loss of ATPase activity) of the rod is nearly one rod per myosin (Figure 5). In separate experiments, the inhibition of actomyosin ATPase by myosin rods could be detected over a wide range of actin concentrations. Inhibitory effects by light meromyosin and squid paramyosin have been previously reported (Konno et al., 1988). Similar effects by rod on regular myosin filaments are shown in Figure 6. The double-reciprocal plots of Figure 6 and the elevation of K_m values by rods indicate that their incorporation into the filament decreases the affinity of myosin for actin and does not affect the V_{max} values. Several control experiments were done to ensure that rod inhibited actomyosin ATPase activity only by increasing the spacing between myosin heads on the filament. First, the exclusive formation of myosin and rod copolymers was verified by analytical ultracentrifugation. Single sedimenting species (15.6 S) were observed for mini-

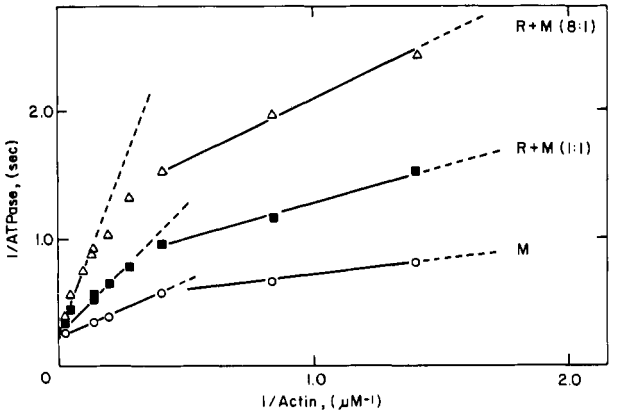

FIGURE 6: Double-reciprocal plots of the actomyosin ATPase activities of myosin and myosin rod cofilaments. Myosin filaments (○), cofilaments composed of equal ratios of myosin and myosin rod (■), and cofilaments composed of one part myosin and eight parts myosin rod (△) were assayed for actomyosin ATPase activities at 25 °C in 40 mM KCl, 10 mM Tris, pH 8.0, 3 mM $MgCl_2$, 2 mM ATP, 0.44 μM myosin, and 0–70 μM actin. The two phases of the plot, at low and high actin concentrations, are indicated by separate lines. All curves extrapolate to a V_{max} value of 5 s^{-1} (at high actin concentrations) with values of 4, 8, and 25 μM for K_m of myosin and 1:1 and 1:8 myosin to rod, respectively.

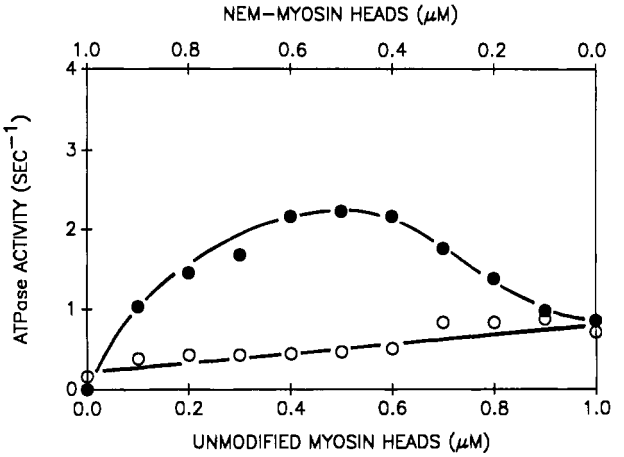

FIGURE 7: Actomyosin ATPase activities of copolymers of intact myosin and NEM-labeled myosin minifilaments. Minifilaments of unmodified myosin and NEM-(SH_1)-myosin (modified to 99% loss of EDTA ATPase activity) were mixed at appropriate molar ratios and allowed to exchange for 30 min at 25 °C prior to the addition of 8 μM actin and ATPase assays (●). Alternatively, minifilaments of unmodified myosin and NEM-(SH_1)-myosin were assayed separately with myosin heads at concentrations ranging between 0.1 and 1.0 μM, and their actin-activated ATPase activities were added to yield *theoretical* activities for mixtures of noninteracting minifilaments (○). The actomyosin ATPase activities were assayed at 25 °C in 20 mM KCl, 5 mM citrate–Tris, pH 8.0, 2 mM $MgCl_2$, 2 mM ATP, 8 μM actin, and up to 1 μM myosin heads.

filament copolymers of rod (1 mg/mL) and myosin (1 mg/mL; data not shown). When run separately, rod and myosin minifilaments sedimented as 14.4S and 22.1S species, respectively. Viscosity changes due to the presence of rod particles in the mixture did not influence the kinetic properties of myosin heads. Rod (8-fold molar excess over S-1) was found to have no effect on acto–S-1 ATPase activities (data not shown).

(B) Elevation of Actomyosin ATPase Activity by Incorporation of SH_1-Labeled Myosin into Myosin Filaments and Minifilaments. Figure 7 illustrates how large can be the elevation of actomyosin ATPase activity due to the modification of SH_1 on a fraction of the myosin heads in the minifilament. Only the actual ATPase activities averaged over all modified and unmodified heads in the minifilaments are plotted in Figure 7. If the same data are expressed in terms of actomyosin ATPase activities per unmodified heads, the observed activations are even greater. For example, in a copolymer containing 10% unlabeled and 90% SH_1-labeled myosins, the actomyosin ATPase activity per unmodified head is 10-fold greater than that of intact myosin in the absence of SH_1-labeled protein. It is also important to note that the minifilament copolymers (Figure 7) were formed with myosin that had been modified to near-completion on SH_1. Therefore, the loss of actomyosin ATPase activity in pure NEM-labeled myosin and the elevation of ATPase activities in the copolymers were due to the same modifications on myosin. Strikingly similar activation of myosin ATPase activity in copolymers of NEM-modified and unmodified *Acanthamoeba* myosin II has been reported (Atkinson et al., 1989). One possible explanation for the activation of the actomyosin ATPase activity of unmodified myosin heads by adjacent (on the filament) labeled myosins could be the higher affinity of the latter for actin in the presence of ATP.

The increased affinity of SH_1-modified myosin heads for actin in the presence of ATP was first indicated by double-reciprocal plots of their actin-activated ATPase activities (Figure 3; Mulhern et al., 1975). Light-scattering measurements carried out in this work (data not shown) verified that the binding of SH_1-labeled S-1 to actin was stronger than that of unmodified S-1, though not as much as found by kinetic K_m determinations (Mulhern et al., 1975). The binding constants of S-1 modified with PM ($K_a = 8 \times 10^3$ M^{-1}) and NEM ($K_a = 16 \times 10^3$ M^{-1}) to actin were by 30% and 160%, respectively, higher than for the unlabeled S-1 ($K_a = 6 \times 10^3$ M^{-1}) in the presence of ATP. On this basis, and assuming a simple binding model for actin activation of ATPase activities, higher ATPases could be expected in partially SH_1-labeled myosin minifilaments than in unlabeled myosin minifilaments. Interestingly, the elevation of actomyosin ATPase activity in partially PM-modified minifilaments was very similar to that in partially NEM-modified minifilaments (data not shown). The actomyosin ATPase activities were less elevated for regular filaments modified with either NEM or PM (data not shown).

Discussion

Although the inhibition of actin-activated ATPase activity in SH_1-labeled myosin fragments is well established (Silverman et al., 1972; Mulhern et al., 1975; Mulhern & Eisenberg, 1976, 1978), the effect of SH_1 labeling on actomyosin ATPase has been controversial. The data presented here offer an explanation for the discrepancies found in the literature on the effects of SH_1 modification on actomyosin ATPase activity. It is shown that cooperative effects in the filament allow the unmodified heads to compensate for the loss of actomyosin ATPase activity in the SH_1-labeled myosin heads. Thus, while near-stoichiometric labeling of SH_1 groups in myosin filaments resulted in near-extinction of the actin-activated ATPase activity (Mulhern et al., 1975; Mulhern & Eisenberg, 1976), fractional modification of these groups had little or no effect on the actomyosin ATPases (Mendelson et al., 1975; Thomas et al., 1980; Thomas & Cooke, 1980; Fajer et al., 1988). In other studies, in which myosin was modified to various extents (Belágyi & Röth, 1987; Titus et al., 1989), or low amounts of actin were used (Seidel, 1973; Yamaguchi et al., 1973), different effects of SH_1 labeling on actomyosin ATPase activities were found.

The correlation between the loss of actomyosin ATPase activity and the loss of shortening (Figure 2) indicates that

the modified myosin head does not undergo a normal cross-bridge cycle and that the unmodified heads can compensate for this loss. The contraction of actomyosin threads made with myosin labeled on SH_1 to varying extents has been measured in an earlier work (Harrington et al., 1975). In that study, the loss of contraction relative to the extent of modification was similarly indicative of cooperative interactions between myosin heads. Although the loss of contractility in actomyosin threads (Harrington et al., 1975) was slightly more rapid than in myofibrils (Figure 1), this variance may be accounted for by the structural differences between actomyosin threads and myofibrils or by the different methods for assaying contractility (Cooke, 1986).

It was previously suggested that the loss of shortening in labeled myofibrils was not due to labeling SH_1 but was instead due to other modifications on myosin or elsewhere in the myofibrils (Crowder & Cooke, 1984). Several lines of evidence, however, indicate that modification of SH_1 is sufficient to account for the observed loss of contractility: (i) The loss of shortening parallels the loss of actin-activated ATPase activity of myosin both in the myofibrils and in solution with purified actin (Figure 2). (ii) The modification of SH_1 groups in relaxed myofibrils is quite specific (Figure 4; radiolabeling studies, data not shown). (iii) Myosin labeled on SH_1 in solution also loses its ability to support contraction of actomyosin threads in a manner indicative of cooperative interactions (Harrington et al., 1975). (iv) Myosin and S-1 extracted from modified myofibrils have different actin-activated ATPases. This shows that the assembly of myosins into filaments is responsible for the ATPase properties of the modified myofibrils and myosin. (v) Modifications of other sulfhydryl groups in myosin cannot account for the initially slow and then rapid loss of actomyosin ATPases with the progressive loss of the K^+ (EDTA) ATPase activity (Figure 2). Had the inactivation of actomyosin ATPase activity depended on the modification of an unknown thiol on S-1 (which lags after SH_1 modification), then the ATPase activities of copolymers of inactivated and intact myosin should have varied linearly with the content of the intact myosin in the filament. This linear relationship was not observed when 96% modified myosin and unlabeled myosin were used to form copolymers for ATPase assays done in conjunction with Figure 2.

One intriguing explanation for the inhibition of actin-activated ATPase activity and the loss of contractility upon SH_1 modification is that this region may be an important coupling element between the nucleotide and actin binding sites. This speculation is supported by observations on the reciprocal effects on each other of SH_1, actin binding, and nucleotide binding sites on S-1 (Cooke, 1986; Duong & Reisler, 1989). Significantly, the ability of ATP to dissociate actomyosin is greatly reduced when the region around SH_1 is selectively cleaved (Chaussepied et al., 1986). Reduction of the rigor binding (Greene & Eisenberg, 1980) and enhancement of the binding to actin in the presence of ATP upon SH_1 modification are also observed.

The mechanism by which cooperativity occurs at the filament level may be more complex than could be deduced from simple binding considerations. Decreases in the binding affinity of minifilaments for actin correlate with changes in the actn-activated ATPase activities. At the same time, the V_{max} values per myosin head are the same regardless of the fraction of rod incorporated into myosin filaments (Figure 6). In the more complex system, in which the affinity of myosin minifilaments for actin in the presence of ATP is increased by the modified heads, the elevation of ATPase activity appears greater than the change in binding. NEM-modified S-1 has a 2.6-fold higher binding constant for actin in the presence of ATP than unmodified S-1 (data not shown). Yet when the fraction of modified myosin per minifilament is high, the ATPase activity of unmodified heads is at least 10-fold higher than that in homogeneous, unmodified myosin minifilaments (calculated from Figure 7). When PM-modified myosin is used, the activations of actomyosin ATPase activity are similar, even though the affinity of PM-modified S-1 for actin is about half that of NEM-modified S-1 (data not shown). Also, it can be calculated from Figure 2 that when most of the myosin is labeled on SH_1, the V_{max} per unmodified head is elevated by approximately 2-fold in comparison to myosin in unmodified filaments (Figure 2). The V_{max} value is extrapolated to an infinite concentration of actin and should, therefore, be independent of the affinity of myosin for actin. It can be concluded that cooperativity in the labeled myosin filament is determined not only by actomyosin binding.

Several studies have examined the issue of cooperative interactions between myosin heads. The inactivation of single heads of myosin by nonhydrolyzable ATP analogues caused no apparent change in the actomyosin ATPase activity of unmodified heads (Tokiwa & Morales, 1971) or loss of tension in the fiber per unmodified head (Wilson et al., 1990). Cooperative interactions between myosin heads have been detected upon modification of fibers with a dithiol cross-linking reagent, pPDM (Chaen et al., 1986); however, extensive intermolecular cross-linking of myosin under these conditions casts doubts on such measurements (Hu et al., 1988). Superprecipitation experiments also suggested cooperative interactions in myosin (Tokiwa & Morales, 1971; Yamaguchi et al., 1973; Sekine & Yamaguchi, 1966; Margossian & Lowey; 1973; Gadasi et al., 1974), but the conclusions of these studies have been questioned, since structural changes in the actomyosin can have substantial effects on superprecipitation measurements (Cooke & Franks, 1978). The use of one-headed myosin in motility assays (Harada et al., 1987) and actomyosin threads (Cooke & Franks, 1978) revealed little change in the velocity of shortening or the force generated per myosin head, respectively. The results are understandable, despite the different affinities for actin of one- and two-headed myosins, because of the near-V_{max} conditions of these assays.

Several speculative suggestions can be made to explain how modified myosins might enhance the activity of neighboring intact myosins in the filament. We will focus on only a few. According to a *proximity* explanation, SH_1 modification causes conformational changes in myosin filaments which enable an abnormally close proximity in binding between the myosin and actin filaments and thus lead to higher activity of the unmodified myosin heads. The close proximity of actin to myosin in myofibrils may be responsible for the higher single-turnover rates of myofibrils compared to S-1 in the presence of high concentrations of actin (Sleep, 1981). The so-called "superactivation" of acto–S-1 ATPase activity induced by zero-length cross-linking of actin to S-1 might also be explained by a close proximity between actin and S-1 (Mornet et al., 1981). Another explanation, that of *induced binding*, postulates that the binding of modified myosin heads induces structural changes along the actin filament which increase the binding of other myosin heads. Such speculation is prompted by the literature data. Conformational changes in the actin filament due to the binding of S-1 have been reported by Ando (1989). Evidence that not all myosin heads are optimally activated in myosin filaments comes from a lower V_{max} for myosin filaments than for S-1 (Pope et al., 1981). Studies in

fibers also indicate at least two populations of myosin heads seeing different effective actin concentrations (Assulin et al., 1986; Pate & Cooke, 1988; Fajer et al., 1988). These results suggest that the mechanism of increasing myosin activity in the filament may involve at least two processes: recruitment of myosin heads which are inaccessible to the actin filament and increased activation of myosin heads which are already exposed to actin. The models suggested above are not mutually exclusive, and contributions from several different mechanisms could be responsible for the observed results.

On the basis of solution studies, and irrespective of cooperative effects in the myosin filaments, the modification of SH_1 is expected to have strong inhibitory effects on the contractile functions of myosin in the fiber. Since the modification of SH_1 with spin-labeled or fluorescent probes has been used for the examination of crossbridge behavior in fibers [for a review, see Thomas (1987)], it is important for such studies that the labeled heads undergo normal crossbridge cycling. Other work has also raised questions about the use of SH_1 probes in evaluating crossbridge behavior: (i) The rate constants of several steps in ATPase hydrolysis and the equilibrium constant of the hydrolysis step are affected by SH_1 modification (Sleep et al., 1981; Barnett & Thomas, 1987). (ii) Labeling SH_1 interferes with calcium regulation of actomyosin ATPase activity (Titus et al., 1989). (iii) The change in dichroism of fluorescent probes on SH_1 has been found to correlate with nucleotide binding, while almost no change is observed during the development of tension (Tanner et al., 1990). Thus, considerable caution should be used in evaluating studies with spectroscopic probes on SH_1.

Caution is needed also because straightforward interpretation of SH_1 modification studies in fibers and in solution is complicated by several factors: (i) Different reagents may depress the actomyosin ATPases to somewhat different extents (Mulhern & Eisenberg, 1976; Svensson & Thomas, 1986). (ii) It is difficult, if not impractical, to achieve greater than 95% modification of SH_1 groups and yet retain good specificity of the reaction. (iii) Different ATP turnover rates in fibers and for acto-S-1 in solution raise questions about the accessibility of myosin heads to actin in the fiber and the mechanism of their interaction.

In conclusion, the loss of shortening upon modification of myofibrils under relaxed conditions parallels the loss of actomyosin ATPase activities of the modified myofibrils and the myosin extracted from them. The loss of actin-activated ATPase activities of S-1 extracted from the modified myofibrils is directly proportional to the extent of SH_1 labeling, yet actomyosin ATPase activities remain at high levels when up to 50–60% of the myosin heads are labeled on SH_1. Complex interactions within the partially modified myosin filaments and with actin cause elevation of the actomyosin ATPase activity, even under V_{max} conditions, of unmodified heads over that of intact myosin in the absence of modified protein. Thus, modification of the SH_1 on myosin alone is sufficient to account for the altered properties of modified myofibrils. This suggests similar catalytic properties for SH_1-modified crossbridges in myofibrils and SH_1-labeled S-1 or myosin in solution and calls into question the nature of the modified crossbridge cycle.

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