# Actin and Temperature Effects on the Cross-Linking of the SH1-SH2 Helix in Myosin Subfragment 1

Lisa K. Nitao and Emil Reisler
Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, California 90095 USA

ABSTRACT Past biochemical work on myosin subfragment 1 (S1) has shown that the bent $\alpha$-helix containing the reactive thiols SH1 ($Cys^{707}$) and SH2 ($Cys^{697}$) changes upon nucleotide and actin binding. In this study, we investigated the conformational dynamics of the SH1-SH2 helix in two actin-bound states of myosin and examined the effect of temperature on this helix, using five cross-linking reagents that are 5–15 Å in length. Actin inhibited the cross-linking of SH1 to SH2 on both S1 and S1.MgADP for all of the reagents. Because the rate of SH2 modification was not altered by actin, the inhibition of cross-linking must result from a strong stabilization of the SH1-SH2 helix in the actin-bound states of S1. The dynamics of the helix is also influenced by temperature. At 25°C, the rate constants for cross-linking in S1 alone are low, with values of $\sim$0.010 $min^{-1}$ for all of the reagents. At 4°C, the rate constants, except for the shortest reagent, range between 0.030 and 0.070 $min^{-1}$. The rate constants for SH2 modification in SH1-modified S1 show the opposite trend; they increase with the increases in temperature. The greater cross-linking at the lower temperature indicates destabilization of the SH1-SH2 helix at 4°C. These results are discussed in terms of conformational dynamics of the SH1-SH2 helix.

## INTRODUCTION

Muscle contraction occurs as a result of the cyclic interactions between myosin, actin, and nucleotides. During the ATPase cycle, regions in the catalytic domain of the myosin head are thought to undergo structural changes, which result in the movement of the lever arm (Fisher et al., 1995; Rayment, 1996; Gulick et al., 1997; Dominguez et al., 1998; Houdusse et al., 1999). It has been proposed that three flexible structural elements in myosin may be involved in coupling the conformational changes of the catalytic domain to the movement of the lever arm (Houdusse et al., 1999). One element of particular interest is the bent $\alpha$-helix containing the reactive sulfhydryls, SH1 ($Cys^{707}$) and SH2 ($Cys^{697}$). Much emphasis has been placed on the SH1-SH2 helix because it has been shown to undergo conformational changes when actin or nucleotides bind to myosin (Burke and Reisler, 1977; Wells et al., 1980; Kameyama and Sekine, 1973). The importance of the flexible nature of this bent helix has also been demonstrated, as mutations of key glycine residues (699 and 710) in the helix have resulted in a perturbation or loss of the myosin motor function (Kinose et al., 1996; Patterson et al., 1997; Batra et al., 1999). In addition, the location of this helix in the structure of S1 places it at or near the fulcrum between the catalytic domain and the lever arm, i.e., in the appropriate position for signal transduction between these domains (Uyeda et al., 1996). Currently there is an even greater need to understand the conformational dynamics of the SH1-SH2 helix. Structural studies of myosin from scallop muscle suggest that the SH1-SH2 helix may adopt different conformations in different nucleotide states (Houdusse et al., 1999). The different conformations of the helix may be associated with specific orientations of the lever arm. However, these structural studies can neither depict the effect that actin binding may have on the SH1-SH2 helix nor fully describe the dynamic nature of this helix.

Cross-linking is a well-established method, often used to probe distances between and within proteins. In myosin, cross-linking has been used to show that the SH1-SH2 helix does indeed undergo conformational changes. In our previous study, a method was devised to separate the cross-linking reaction from the SH1 modification reaction, thereby enabling us to determine the rate constants of SH1-SH2 cross-linking (Nitao and Reisler, 1998). With this method, we were able to measure in kinetic terms the accelerating effect of nucleotide binding on the helix cross-linking. The rates of SH1-SH2 cross-linking obtained for reagents that are 5–15 Å in length were correlated with the probabilities (or frequencies) at which different conformational states of the SH1-SH2 helix (different SH1 to SH2 distances) are occupied in the presence of MgADP, MgATP$\gamma$S, and MgATP. The observed increases in cross-linking rates are thought to originate from the destabilization of the SH1-SH2 helix by nucleotides. The most striking destabilization of this helix was observed in the S1.MgATP$\gamma$S complex, which is believed to mimic the S1.MgATP state in the cross-bridge cycle. Because such a destabilization implies orientational disorder of the lever arm relative to the catalytic domain in the S1.MgATP state, assuming a rigid-body like structure of the lever arm, it has become important to describe the SH1-SH2 helix structure in other states of myosin in the cross-bridge cycle.

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Address reprint requests to Dr. Emil Reisler, Department of Chemistry and Biochemistry, University of California, 405 Hilgard Avenue, Los Angeles, CA 90095. Tel.: 310-825-2668; Fax: 310-206-7286; E-mail: reisler@ewald.mbi.ucla.edu.



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In this study, we examined the acto-S1 and acto-S1.MgADP states. Actin appears to have a stabilizing effect on the overall structure of S1, in both the absence and presence of MgADP (Highsmith and Jardetzky, 1980; Nikolaeva et al., 1996). In the SH1-SH2 helix specifically, the binding of actin to S1 induces several structural changes. When actin is bound, SH1 is protected from modification by alkylating reagents (Duke et al., 1976; Cartoux et al., 1992; Polosukhina and Highsmith, 1997; Hiratsuka et al., 1998). The addition of MgADP to acto-S1 results in a slight decrease in the protection of SH1 (Hiratsuka et al., 1998). Because of the inhibition of SH1 modification in the acto-S1 complex the effect of actin on the SH1-SH2 helix and its cross-linking rate, especially in the absence of MgADP, could at best be estimated but not accurately determined (Polosukhina and Highsmith, 1997). Therefore, in this work, the bifunctional reagents were attached to SH1 on isolated S1, and such premodified S1 was then used to determine what effect actin alone, and actin and MgADP, may have on the SH1-SH2 helix.

In addition to the effects of actin, we also explored the effect of temperature on the dynamics of the SH1-SH2 helix. This is of particular interest because S1 crystals have been grown at low temperatures. Furthermore, although past studies suggest that S1 may adopt at least two temperature-dependent conformations (Shriver, 1986; Aguirre et al., 1989), these conformations were not linked to the transitions in the SH1-SH2 helix. To examine the role temperature may play in the stability of the helix, the cross-linking experiments were performed at two temperatures (4°C and 25°C) for the various nucleotide- and actin-bound states. These results are discussed in terms of the flexibility of the SH1-SH2 helix.

## MATERIALS AND METHODS

### Reagents

*N*-Ethylmaleimide (NEM), ADP, and Sephadex G-50 were purchased from Sigma (St. Louis, MO). *N,N′*-1,2-phenylene dimaleimide (oPDM) and *N,N′*-1,4-phenylene dimaleimide (pPDM) were from Research Organics (Cleveland, OH). *N*-Phenylmaleimide (PM), *N,N′*-1,3-phenylene dimaleimide (mPDM), napthalene-1,5-dimaleimide (NDM), and 1,1′-(methylenedi-4,1-phenylene)bismaleimide (BM) were from Aldrich (Milwaukee, WI). Adenosine-5-(3-thio)triphosphate (ATPγS) was obtained from Boehringer Mannheim (Mannheim, Germany).

### Proteins

Myosin and actin from rabbit psoas muscle were prepared according to the method of Godfrey and Harrington (1970) and Spudich and Watt (1971), respectively. Subfragment-1 (S1) was prepared by chymotryptic digestion of myosin filaments as described (Weeds and Pope, 1977). The concentration of S1 and actin was determined spectrophotometrically by using extinction coefficients of $E^{1\%}_{280} = 7.5\ \text{cm}^{-1}$ and $E^{1\%}_{292} = 11.5\ \text{cm}^{-1}$. NEM-modified actin (at $Cys^{374}$) was prepared by adding a 10-fold excess of NEM to G-actin for 3 h. The reaction was stopped with the addition of 1.0 mM dithiothreitol (DTT). The extent of modification of $Cys^{374}$ was determined by monitoring the loss of quenching of tryptophan fluorescence by $Cu^{2+}$, as described previously (Lehrer et al., 1972). Under these conditions, ~95% of the actin was modified (data not shown).

### Cosedimentation assays

The binding of SH1-modified S1 to F-actin or NEM-F-actin in the absence or presence of 1.0 mM MgADP was measured using the cosedimentation assays as described previously (Miller and Reisler, 1995). S1 (12 μM) was reacted with a fourfold excess of PM in 10 mM KCl, 10 mM imidazole, at pH 7.0 at 4°C. After 30 min, the reaction was stopped with the addition of 1.0 mM DTT. Under these conditions, ~80% of the SH1 groups were modified (data not shown). To remove the DTT, SH1-PM S1 was dialyzed overnight in 10 mM KCl, 10 mM imidazole, at pH 7.0. This SH1-PM S1 (8.0 μM) was then incubated for 30 min at room temperature in the presence of actin (25 μM) and 1.0 mM $MgCl_2$. In one sample, 1.0 mM MgADP was also added. The samples were centrifuged at room temperature in a Beckman airfuge at 140,000 × *g* for 20 min. The resuspended pellets were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970). The intensities of the actin and S1 bands stained by Coomassie blue were quantified by scanning the gels on an AGFA scanner and analyzing the densities of the S1 and actin bands with the use of Sigmagel. The cosedimentation assays were also carried out using SH1-NEM S1 (10 μM) (modification described later) and NEM-F-actin (30 μM) in the presence and absence of 1.0 mM MgADP.

### ATPase activities

$Ca^{2+}$- and EDTA- ($K^+$-) ATPase activities of S1 were determined, as described before, at 37°C (Fiske and Subbarow, 1925; Nitao and Reisler, 1998). The $Ca^{2+}$-ATPase assay solution contained 600 mM KCl, 50 mM Tris-HCl (pH 7.6), 5.0 mM $CaCl_2$, and 2.0 mM ATP. The EDTA-ATPase assay solution contained 444 mM KCl, 50 mM histidine, 50 mM Tris-HCl (pH 7.6), 5.0 mM EDTA, and 2.0 mM ATP.

### Cross-linking rate determination

As in the previous work (Nitao and Reisler, 1998), all cross-linking reactions were carried out in three steps: modification of SH1 with a bifunctional reagent, removal of excess reagent from SH1-labeled sample, and monitoring of the cross-linking reaction in the presence or absence of ligands. In the first step, S1 (12 μM) was reacted with cross-linking reagents (18 μM) at 4°C. For oPDM reactions, 1.0 mM MgADP was also added. For the other reagents, the modification reactions progressed sufficiently fast, and the addition of nucleotide was not necessary. The time of modification for each reagent was selected so as to yield the largest possible experimental range for subsequent measurements of the SH1-SH2 cross-linking reactions. The conditions of these reactions and all of the control experiments were the same as described previously (Nitao and Reisler, 1998). In most cases, between 30% and 50% of S1 was modified in the first step of the reaction. The amount of S1 modified was determined by measuring the EDTA ATPase activity. In the second step, after SH1 was modified, excess reagent was removed by centrifugation on Sephadex G-50 columns equilibrated with 10 mM KCl, 10 mM imidazole (pH 7.0). In the third step, the cross-linking reaction was monitored. After the sample was split into two equal parts, ligand was added to one of the samples. The other sample was the control, in which the cross-linking of S1 alone was monitored to yield the cross-linking rate, $k_2$. For reactions with F-actin, the SH1-labeled sample (~8 μM) was briefly incubated with actin (25 μM), which had been polymerized by 1.0 mM $MgCl_2$ at room temperature. For reactions with nucleotide, 1.0 mM MgADP or 1.0 mM MgATPγS was added. For the reactions at 4°C, the incubations of S1 with ligands were done on ice. The reactions at 25°C were all done in a water bath. At

selected time points, aliquots of the cross-linking reaction were withdrawn to stop the reaction (with 1.0 mM DTT) and to measure the $Ca^{2+}$- and EDTA-ATPase activities. ATPase activities were analyzed as described previously to calculate the cross-linking rate constants for all of the reagents in the presence of nucleotides ($k_{2N}$), actin ($k_{2A}$), and actin and MgADP ($k_{2AN}$) (Nitao and Reisler, 1998).

### SH2 modification rates

The determinations of SH2 modification rates were made as described previously (Nitao and Reisler, 1998). SH1 was modified by the addition of a fivefold excess of NEM over S1 (20–25 $\mu$M). After 90 min, the reaction was terminated with 1.0 mM DTT. All SH1 modification reactions were carried out in 10 mM KCl, 10 mM imidazole (pH 7.0) at 4°C. SH1-NEM S1 was then dialyzed overnight in 10 mM KCl, 10 mM imidazole (pH 7.0) to remove excess reagent and DTT. SH2 was modified by the addition of a fourfold excess of a cross-linking reagent (oPDM, mPDM, pPDM, NDM, BM in dimethylformamide) to S1 (10 $\mu$M). The modification reactions were performed at 4°C or 25°C in the absence or presence of ligands. For the reactions with NEM-F-actin, SH1-NEM S1 was preincubated with 30 $\mu$M NEM-actin and 1.0 mM $MgCl_2$. In the case of nucleotides, 1.0 mM MgADP or 1.0 mM MgATP$\gamma$S was added. To prevent aggregation in the reactions at 25°C, 0.2 M sucrose was added to the solutions. At various time points, the reactions were quenched in aliquots with 1.0 mM DTT, and the $Ca^{2+}$-ATPase activity of each sample was measured to monitor the extent of SH2 modification. As SH2 was modified, the $Ca^{2+}$-ATPases decreased. The $Ca^{2+}$-ATPase activities of the modified samples were plotted versus reaction time to determine the first-order rate constants of SH2 modifications.

## RESULTS

### Binding of actin to SH1-modified S1

One aim of this study has been to examine the effects that actin and actin/MgADP have on the stability of the SH1-SH2 helix by monitoring the rates of cross-linking of these thiols. Such experiments require that actin will be bound to S1, which is labeled at the SH1 with a bifunctional reagent. Past biochemical work has shown that the presence of MgADP and modifications of S1 at SH1 may reduce the binding affinity of actin to S1 (Schwyter et al., 1989). Cosedimentation assays were performed to determine the conditions under which most of the modified S1 is bound to actin, so that the effect of actin on the flexibility of the SH1-SH2 helix can be determined. This goal was achieved when an approximately threefold molar excess of actin over S1 was used in the reactions and by keeping the salt concentration of the buffers low (10 mM).

In the cross-linking experiments, SH1 was first modified by the cross-linking reagents, then excess reagent was removed, and finally actin was added to determine its effect on the cross-linking. To mimic the S1 used in the cross-linking experiments, SH1 was modified by the monofunctional equivalent of the cross-linking reagents, phenylmaleimide (PM). In the absence of MgADP, all of the SH1-PM S1 (8.0 $\mu$M) was bound to actin (25 $\mu$M). In the presence of 1.0 mM MgADP, ~95% or more of the SH1-PM S1 remained bound to actin (data not shown). These results indicate that under such conditions, which were used in the cross-linking experiments, most if not all of the modified S1 was bound to actin, in both the absence and presence of MgADP.

In the experiments done to determine the rate of SH2 modification, both S1 (at SH1) and actin (at $Cys^{374}$) were modified with NEM before the SH2 reaction. In the binding assays with NEM-actin, most (more than 95%) of the SH1-NEM S1 (10 $\mu$M) was bound to NEM-actin (30 $\mu$M). In the presence of both NEM-actin and MgADP, ~90% or more of the SH1-NEM S1 was found to be bound to NEM-actin (data not shown). These results indicate that most of the SH1-NEM S1 would be bound to NEM-actin during the modification reaction of SH2.

### Effect of actin on the rates of cross-linking and SH2 modification of S1 and S1.MgADP at 25°C

The effects of actin on SH1-SH2 cross-linking on S1 and S1.MgADP are shown in Table 1 in the form of ratios of cross-linking rate constants for the reactions in the presence and absence of actin, $k_{2A}/k_2$, and in the presence and absence of actin and MgADP, $k_{2AN}/k_2$. Along with these values, the individual cross-linking rate constants for S1 alone, $k_2$, are also shown for each of the cross-linking reagents. The cross-linking rate constants in the presence of

**TABLE 1 Ratio of cross-linking rate constants in the presence and absence of actin and MgADP (25°C)**

| Reagent | Cross-linking span (Å) | $k_2$ ($min^{-1}$) | $k_{2A}/k_2$ | $k_{2AN}/k_2$ |
|---|---|---|---|---|
| oPDM | 5.2–7.8 | 0.010 ± 0.003 | ~0 | ~0 |
| mPDM | 9.6–11.5 | 0.014 ± 0.002 | ~0 | ~0 |
| pPDM | 12.1–12.4 | 0.013 ± 0.003 | ~0 | ~0 |
| NDM | 12.4–12.9 | 0.006 ± 0.002 | 0.1 ± 0.1 | 0.4 ± 0.1 |
| BM | 14.9–15.4 | 0.014 ± 0.002 | 0.2 ± 0.1 | 0.4 ± 0.1 |

All rate constants were determined from semilogarithmic plots of the $Ca^{2+}$-ATPase activity of modified S1 versus cross-linking time, as described previously (Nitao and Reisler, 1998). Each rate is the mean value of at least two independent experiments. The ranges of cross-linking distances for each reagent reflect the possible attachments of the sulfhydryls to the maleimide group in the different conformers of the reagents. $k_2$ represents the rate constant of cross-linking in the absence of actin or MgADP. $k_{2A}/k_2$ is the ratio of cross-linking rate constants of S1 in the presence of 25 $\mu$M actin and S1 alone. $k_{2AN}/k_2$ is the ratio of cross-linking rate constants of S1 in the presence of 25 $\mu$M actin and 1.0 mM MgADP versus S1 alone.

actin alone, $k_{2A}$, and actin and MgADP, $k_{2AN}$, are not presented; however, they can be calculated by multiplying the $k_2$ values by the $k_{2A}/k_2$ or $k_{2AN}/k_2$ values, respectively, for each of the reagents. From the results, it is evident that the binding of actin to S1 inhibits SH1-SH2 cross-linking for all of the reagents at 25°C. This can be observed in Fig. 1 for one representative reagent, pPDM. Because there were no significant changes for more than 15 min in the ATPase activities for the cross-linking reactions using oPDM, mPDM, and pPDM, the rates of cross-linking in the presence of actin were considered to be almost completely inhibited for these reagents. For NDM and BM, the cross-linking rates were inhibited by 80–90% compared to those for the control S1. For all of the reagents tested, the addition of MgADP to the acto-S1 complex did not alleviate the inhibition of cross-linking.

One possibility that had to be considered is that the binding of actin to S1 may be activating cross-linking of the reagent on SH1 to another group rather than inhibiting the reaction with SH2. Cross-linking to other cysteines on S1 for reagents attached to either SH1 or SH2 has been reported previously (Mornet et al., 1985; Chaussepied et al., 1986). To verify that the effect of actin is an inhibition of cross-linking to SH2, control experiments were performed for both oPDM and pPDM. After the cross-linking reaction in the presence of actin was monitored for 30 min, 1.0 mM MgATPγS was added to the reaction to dissociate actin from S1. When MgATPγS is bound to S1, the cross-linking reaction for both reagents should be accelerated if the reagents attached to SH1 have not yet reacted with another residue on S1. This was indeed observed for both reagents. Fig. 2 depicts the results obtained for pPDM. During the initial 30 min of the cross-linking step of the reaction, there was no apparent change in the $Ca^{2+}$-ATPase activity of S1. Upon the addition of MgATPγS, there was a rapid decrease in $Ca^{2+}$-ATPase activity, indicating that cross-linking to SH2 was occurring. Therefore, the inhibition of cross-linking observed when actin is present is due to the inhibition of cross-linking to SH2 and not to the loss of the reagent to a competing reaction.

To eliminate any possible differences in the reactivity of the reagents toward SH2, we also determined the rate constants of SH2 modification in SH1-NEM S1 in the presence and absence of actin and actin and MgADP. The effects of actin and actin and MgADP binding on SH2 modification by pPDM are shown in Fig. 3. The results for this and all other SH2 reactions are summarized in Table 2. They indicate that actin does not affect the rate of SH2 modification for any of the reagents. This suggests that under the employed conditions, the SH2 group is available for cross-linking to all of the reagents. In the presence of both actin and MgADP, the results were surprisingly different. The SH2 modification rates were inhibited by 70% for all of the reagents, indicating that at 25°C, the SH2 group may not be

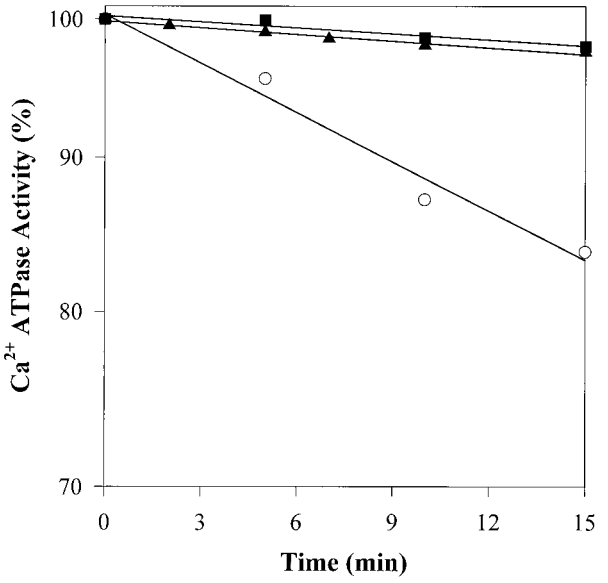


FIGURE 1 Semilogarithmic plots of pPDM cross-linking of S1 in the actin-bound states versus time at 25°C. The cross-linking reactions in S1 with pPDM attached to SH1 were monitored in the absence (○) and presence of 25 μM F-actin (■) or 25 μM F-actin and 1.0 mM MgADP (▲). Although only the initial three data points are shown for the plot in the absence of actin, all of the data points (taken up to 25 min) are well-fitted by the same curve. The first-order rate constants for these reactions are 0.013 $min^{-1}$, 0.0015 $min^{-1}$, and 0.0017 $min^{-1}$, respectively.

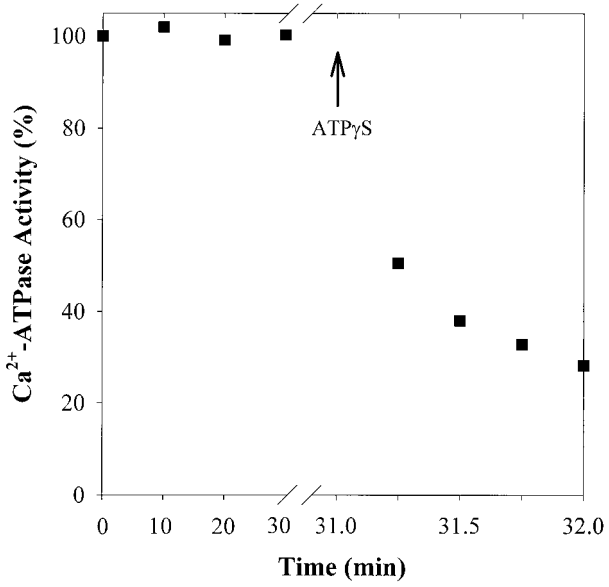


FIGURE 2 Plot of pPDM cross-linking of S1 in the actin-bound state versus time at 25°C. The cross-linking reaction was monitored for 30 min in the presence of 25 μM F-actin (■) via $Ca^{2+}$-ATPase measurements. After minute 31 (indicated by the *arrow*), 1.0 mM MgATPγS was added to the reaction mixture. The rapid decrease in the $Ca^{2+}$-ATPase activity of S1 after MgATPγS addition reflects the cross-linking of SH1 to SH2. A similar relief of actin inhibition of SH1-SH2 cross-linking was also observed for oPDM after the addition of MgATPγS.

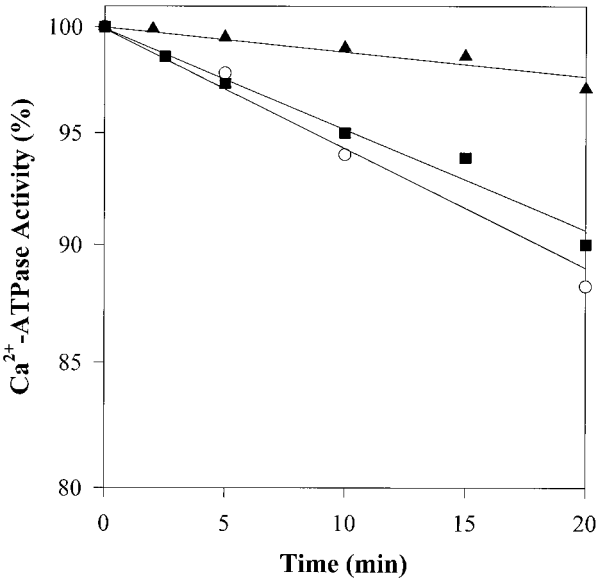


FIGURE 3 Semilogarithmic plots of the pPDM modification of SH2 in the actin-bound states of SH1-NEM S1 versus time at 25°C. The modification reactions were monitored in the absence (○) and presence of 30 $\mu$M F-actin (■) or 30 $\mu$M F-actin and 1.0 mM MgADP (▲). The addition of actin alone did not result in any significant changes in the rate of SH2 modification ($k_2$ = 0.048 min$^{-1}$, $k_{2A}$ = 0.039 min$^{-1}$). Addition of both actin and MgADP resulted in an ~80% inhibition of the SH2 modification rate ($k_{2AN}$ = 0.010 min$^{-1}$).

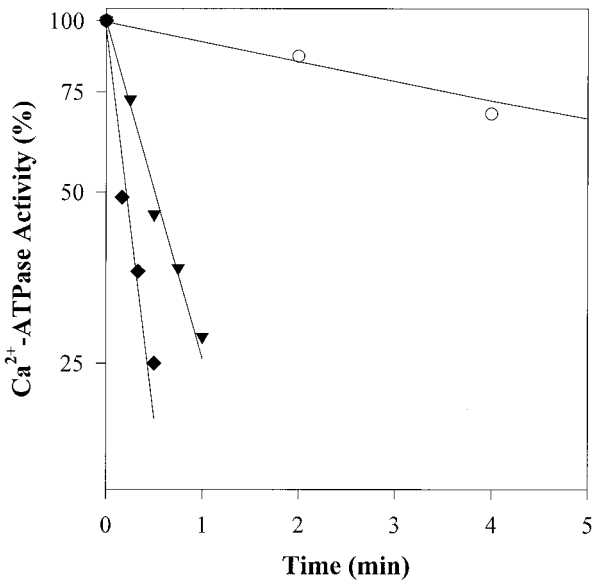


FIGURE 4 Semilogarithmic plots of pPDM cross-linking of S1 in the nucleotide-bound states versus time at 4°C. The cross-linking reactions in S1 with pPDM attached to SH1 were monitored in the absence (○) and presence of 1.0 mM MgADP (▲) or 1.0 mM MgATPγS (◆). The rate constants for these reactions are 0.073 min$^{-1}$, 1.34 min$^{-1}$, and 3.97 min$^{-1}$, respectively. Although only the initial two data points for S1 cross-linking are shown, all of the data points (taken up to 10 min) are well fitted by the same curve.

readily accessible to the reagents present in the medium. Notably, in each group of reactions, with and without actin, the rates of SH2 labeling on SH1-NEM S1 were approximately the same for all of the reagents, with the exception of oPDM.

## Effect of temperature on cross-linking rates

Another aim of this study was to determine the effect of temperature on the flexibility of the SH1-SH2 helix by measuring the cross-linking rates at 4°C and 25°C. The $k_2$ values for all of the reagents at 25°C are shown in Table 1. These values, which range from 0.006 min$^{-1}$ (NDM) to 0.014 min$^{-1}$ (BM), do not vary significantly from reagent to reagent. As can be seen from Table 3, the $k_2$ values determined at 4°C differ to a much greater extent and are approximately fourfold greater than the corresponding rate constants at 25°C (except oPDM). This could indicate that the helix is either more flexible or destabilized, or that SH2 is considerably more reactive at the lower temperature. The latter possibility is rejected because SH2 modification rates on SH1-NEM S1 are much slower at 4°C than at 25°C (Table 4). Therefore, the faster SH1-SH2 cross-linking at the lower temperature must originate from a change in the helix stability.

Table 3 lists both the individual rate constants for cross-linking in the absence and presence of nucleotides at 4°C and the ratios of these rates ($k_{2N}/k_2$), which can be compared to the results previously determined for 25°C (Nitao and Reisler, 1998). In the MgADP- and MgATPγS-bound states, the $k_{2N}$ values at 4°C are, respectively, twofold and 1.5-fold greater, at most, than the $k_{2N}$ values obtained at 25°C (Fig. 4). This suggests that in the nucleotide-bound state, the SH1-SH2 helix is somewhat more destabilized at 4°C than at 25°C. In terms of the $k_{2N}/k_2$ ratios, there were no

**TABLE 2 Effect of actin on the SH2 modification rates in SH1-NEM S1 and SH1-NEM S1.MgADP (25°C)**

| Reagent | $k_2$ (min$^{-1}$) | Ratio of rates | |
|---|---|---|---|
| | | $k_{2A}/k_2$ | $k_{2AN}/k_2$ |
| oPDM | 0.005 ± 0.001 | 1.1 ± 0.05 | 0.2 ± 0.01 |
| mPDM, pPDM, NDM, BM | 0.028 ± 0.010 | 1.0 ± 0.3 | 0.3 ± 0.1 |

In the modification experiments, 30 $\mu$M F-actin was polymerized by 1.0 mM $MgCl_2$, then incubated with SH1-NEM S1 for 5 min before the addition of cross-linking reagents. In the reactions with nucleotide, 1.0 mM MgADP was added to acto-S1 before the addition of cross-linking reagents. All rate constants were determined from semilogarithmic plots of the $Ca^{2+}$-ATPase activity of modified S1 versus time of reaction. At least two independent rate determinations were made for each reagent. For mPDM, pPDM, NDM, and BM, the rate constants were similar and are, therefore, the mean values for the four reagents. The $k_{2A}/k_2$ values represent the ratios of the rate constants of SH2 modification in the presence and absence of actin. The $k_{2AN}/k_2$ values represent the ratios of the rate constants of SH2 modification in the presence and absence of actin/MgADP.

**TABLE 3 Cross-linking rate constants in the presence and absence of nucleotides at 4°C**

| Reagent | $k_2$ ($min^{-1}$) | $k_{2N}$ ($min^{-1}$) | $k_{2N}/k_2$ |
|---|---|---|---|
| | | MgADP | |
| oPDM | 0.013 ± 0.001 | 0.04 ± 0.01 | 3 ± 1 |
| mPDM | 0.055 ± 0.004 | 1.01 ± 0.05 | 18 ± 2 |
| pPDM | 0.070 ± 0.005 | 1.30 ± 0.05 | 19 ± 2 |
| NDM | 0.028 ± 0.002 | 0.70 ± 0.03 | 25 ± 3 |
| BM | 0.071 ± 0.006 | 1.12 ± 0.05 | 16 ± 2 |
| | | MgATPγS | |
| oPDM | 0.014 ± 0.002 | 0.06 ± 0.01 | 4 ± 1 |
| mPDM | 0.052 ± 0.008 | 1.58 ± 0.16 | 30 ± 8 |
| pPDM | 0.065 ± 0.004 | 3.86 ± 0.38 | 60 ± 9 |
| NDM | 0.028 ± 0.001 | 1.70 ± 0.11 | 60 ± 6 |
| BM | 0.069 ± 0.004 | 2.11 ± 0.20 | 30 ± 5 |

All rate constants were determined from semilogarithmic plots of the $Ca^{2+}$-ATPase activity of modified S1 versus cross-linking time. Each rate is the mean value of at least three independent experiments. $k_2$ represents the rate constant of cross-linking in the absence of nucleotide. $k_{2N}$ represents the rate constants of cross-linking in the presence of 1.0 mM MgADP or MgATPγS.

significant temperature differences observed for oPDM, the shortest reagent. For this reagent, MgADP or MgATPγS accelerated SH1-SH2 cross-linking severalfold at both 4°C and 25°C (Nitao and Reisler, 1998). For the longer reagents, the accelerations in cross-linking at 4°C (i.e., $k_{2N}/k_2$) ranged from 16- to 25-fold and from 30- to 60-fold for MgADP and MgATPγS, respectively. Although these ratios were smaller than those previously determined at 25°C (between 40 and 60 for MgADP and between 80 and 270 for MgATPγS), it appears that such differences in $k_{2N}/k_2$ ratios at the two temperatures may result mainly from a greater destabilization of the SH1-SH2 helix in the nucleotide-free state.

The effects of actin and actin/MgADP on cross-linking were also determined at 4°C. The cross-linking in the presence of actin was inhibited for oPDM and mPDM by 90% and 40%, respectively, relative to the cross-linking rates of S1 alone. For the longer reagents (pPDM, NDM, and BM), the cross-linking rates were not significantly altered. In the presence of actin and MgADP, the cross-linking rates for oPDM and mPDM remained inhibited, while the rates for the longer reagents were slightly accelerated (approximately twofold). Fig. 5 shows representative results for a cross-linking reaction with pPDM. As the results indicate, the effects of actin and actin/MgADP on cross-linking at 4°C differ from those observed for 25°C, in which the cross-linking was inhibited for all of the reagents in the actin-bound states (see Table 1).

**TABLE 4 SH2 modification rate constants in the presence and absence of nucleotides at 4°C**

| Reagent | $k_2$ ($min^{-1}$) | $k_{2N}$ ($min^{-1}$) | $k_{2N}/k_2$ |
|---|---|---|---|
| | | MgADP | |
| oPDM | 0.0012 ± 0.0003 | 0.047 ± 0.002 | 40 ± 12 |
| mPDM, pPDM, NDM, BM | 0.0056 ± 0.001 | 0.28 ± 0.05 | 50 ± 18 |
| | | MgATPγS | |
| oPDM | 0.0010 ± 0.0002 | 0.046 ± 0.002 | 46 ± 11 |
| mPDM, pPDM, NDM, BM | 0.0053 ± 0.001 | 0.33 ± 0.05 | 62 ± 21 |

All rate constants were obtained from semilogarithmic plots of the $Ca^{2+}$-ATPase activity of modified S1 versus time of reaction. At least two independent modification experiments were carried out for each reagent. For mPDM, pPDM, NDM, and BM, the rate constants were similar and are, therefore, the mean values for the four reagents. $k_2$ represents the rate constant of SH2 modification in the absence of nucleotide. $k_{2N}$ represents the rate constants of SH2 modification in the presence of 1.0 mM nucleotide (MgADP or MgATPγS).

## Effect of temperature on SH2 modification rates

The SH2 modification reactions were also performed at 4°C to help with the interpretation of the cross-linking data. In Table 4, the individual rate constants for SH2 modification and the $k_{2N}/k_2$ ratios are shown for all of the reagents. The $k_2$ values determined at 4°C are approximately sixfold smaller than those at 25°C for all of the reagents (see Tables 4 and 2). These results show that despite the faster SH1-SH2 cross-linking (Tables 1 and 3), the reactivity or accessibility of SH2 in SH1-NEM S1 in the absence of nucleotides is apparently smaller at 4°C than at 25°C. The $k_{2N}$ values in the presence of MgADP and MgATPγS are approximately twofold greater at 4°C (Table 4) than at 25°C (Nitao and Reisler, 1998). The $k_{2N}/k_2$ ratios were between 40 and 60 for

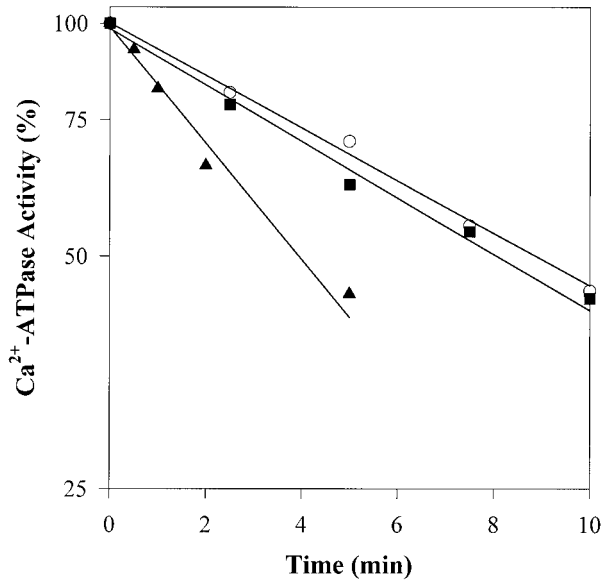


FIGURE 5 Semilogarithmic plots of pPDM cross-linking of S1 in the actin-bound states versus time at 4°C. The cross-linking reactions in S1 with pPDM attached to SH1 were monitored in the absence (○) and presence of 25 μM F-actin (■) or 25 μM F-actin and 1.0 mM MgADP (▲). The addition of actin alone appeared to have no effect on the rate of cross-linking, while the addition of both actin and MgADP increased the cross-linking rate by twofold.

all of the reagents in the MgADP- and MgATPγS-bound states of S1. These values differ from similar ratios measured at 25°C, which ranged between 3 and 8 (Nitao and Reisler, 1998). In the actin-bound states, the effect of actin on the rate of SH2 modification was similar for all of the reagents at both temperatures, in that actin did not appear to affect the modification rate. In the presence of both actin and MgADP, the SH2 modification rates were inhibited at 25°C (Table 2), while these rates were increased by approximately five- to eightfold with the same reagents at 4°C (data not shown).

## DISCUSSION

One of the main goals of the current study has been to determine the conformational dynamics of the SH1-SH2 helix in the strongly bound states of the cross-bridge cycle, acto-S1 and acto-S1.MgADP. These states have a well-defined geometry and ordered appearance in electron microscopy images (Moore et al., 1970; Cooke, 1997) and reveal little, if any mobility of spin probes attached to the light-chain region (Baker et al., 1998; Roopnarine et al., 1998). This is in contrast to the weakly bound states of acto-S1, which appear to be disordered in EM (Walker et al., 1994, 1999; Cooke, 1997). In the previous study, we showed a strong destabilization of the SH1-SH2 helix in S1.MgATPγS, the analog of the S1.MgATP state (Nitao and Reisler, 1998). We also speculated that such a destabilization of the SH1-SH2 helix might give rise to the disorder of the lever-arm region of S1 relative to its catalytic domain if the lever-arm motions could be simplistically viewed as rigid-body movements. The significance of this observation and speculation has been amplified by the recent solution of the atomic structure of scallop S1.MgADP, in which the SH1-SH2 helix appears to be melted. On the basis of structural considerations, Houdusse et al. (1999) concluded that the new S1 structure corresponds to the S1.MgATP state. If that is indeed the case, solution and crystallographic evidence would converge for the first time to identify the S1.MgATP state with a destabilized or melted SH1-SH2 helix. If we assume next that the conformation of this helix is responsible for (or contributes to) the disorder of acto-S1.MgATP, then the strongly bound acto-S1 complexes should show attenuated dynamics of the SH1-SH2 region. Such a conclusion, on actin stabilization of the SH1-SH2 helix, was reached in a previous study (Polosukhina and Highsmith, 1997). However, because in that study the steps of reagent attachment to SH1 and its cross-linking to SH2 were not separated, and the rate of SH1 modification was estimated to be ~0, it is difficult to determine accurately, if at all, the effect that actin has on the cross-linking in such a reaction. Therefore, measurements of the actin effect on the conformation of the SH1-SH2 helix were designed to test more rigorously the expectation that actin stabilizes the helix.

### Stabilization of the SH1-SH2 helix in the actin-bound states

To probe the conformational changes occurring within the helix in the actin-bound states, we first established the conditions for almost complete acto-S1 binding during the cross-linking experiments. The results presented for pPDM (Fig. 1) and all of the cross-linking reagents (Table 1) demonstrated that the cross-linking of SH1 to SH2 is strongly inhibited when actin is bound to S1. Because actin has little effect on the rate of monofunctional modification of SH2 on SH1-NEM S1 by any of the cross-linking reagents (see Table 2), our results point to the stabilization of the SH1-SH2 helix by actin. The reactivation of cross-linking with the addition of MgATPγS to acto-S1 confirms that the actin-induced inhibition of cross-linking is due to the inability of the reagent on SH1 to react with SH2 and not the loss of the reagent to another reaction (Fig. 2). Our results indicate that actin greatly inhibits the ability of these residues to approach each other (within 5–15 Å).

In the presence of both actin and MgADP, the cross-linking of SH1 to SH2 remains inhibited. Because MgADP alone accelerates this reaction (Nitao and Reisler, 1998), the inhibition of cross-linking shows that the effect of actin is dominant, and the helix is stabilized also in the acto-S1.MgADP state. Interestingly, the SH2 modification reactions on acto-S1(SH1-NEM).MgADP reveal local differences around the SH2 residue between acto-S1 and acto-S1.MgADP. Although the SH1-SH2 helix is stabilized in both complexes, the inhibition of monofunctional SH2 modification is observed only for acto-S1.MgADP. The accelerated modification of SH2 on S1.MgADP precludes the possibility that the acto-S1.MgADP result is due to some S1.MgADP free of actin (Nitao and Reisler, 1998).

### Effect of temperature on the SH1-SH2 helix

Another goal of this work was to determine the role of temperature in the stability of the helix. Previous studies have shown that S1 may adopt two different conformations, which are temperature sensitive (Shriver, 1986; Aguirre et al., 1989). The temperature-dependent change in the equilibrium population of these two S1 conformations led to the prediction that SH1-SH2 cross-linking would be faster at low than at high temperatures (Shriver, 1986). Consistent with this, the comparison of the cross-linking reactions reveals significant kinetic differences between 4°C and 25°C. The cross-linking rate constants, $k_2$, are about fourfold greater (except for the shortest reagent, oPDM) and show a greater range of values at 4°C than at 25°C. Yet the modification of SH2 on SH1-NEM S1, using the same reagents, is about sixfold slower at 4°C than at 25°C (Table 4 of this work and Table 2 in Nitao and Reisler, 1998). Together, these strikingly opposite kinetic trends show that in the absence of nucleotides the SH1-SH2 helix is less

stable at 4°C than at 25°C. The lower intrinsic stability of the SH1-SH2 helix at 4°C also results in lower $k_{2N}/k_2$ ratios (Table 3) than those observed for 25°C (Nitao and Reisler, 1998). Clearly, the differences in the stability of the SH1-SH2 helix between the nucleotide-bound and free states of S1 are decreased at the lower temperature. In contrast to this, the differences in the local environment and SH2 reactivity between these states (as measured by $k_{2N}/k_2$ ratios) are greater at the lower temperature.

The above temperature effects make possible a clearer distinction between local changes in the SH2 accessibility and reactivity and the stability of the SH1-SH2 helix. Because the nucleotide-induced changes in this helix are smaller at 4°C than at 25°C, these results raise the possibility that the crystallization process contributes to the trapping of specific helix conformations. It is also conceivable that the stability of the helix and the equilibria between the two S1 conformations depend on the S1 isoform, the presence or absence of the light chains on S1, the truncation point on the S1 heavy chain, and other factors. The successful trapping of the melted SH1-SH2 helix in scallop S1.MgADP crystals (Houdusse et al., 1999) may result from a greater destabilization of this helix in scallop S1 than in *Dictyostelium* S1.

### Alternative view of the SH1-SH2 helix

An alternative depiction of the SH1-SH2 helix has been offered in a recent study by Kliche et al. (1999). These authors utilize a series of aliphatic bifunctional reagents with cross-linking spans ranging from ~2 to 20 Å. They interpret their results as indicating that the separation between SH1 and SH2 is about the same (≥15 Å) in the presence or absence of MgADP. However, there are two reservations regarding this work. First, the longer reagents used in that study have extended -$CH_2$- linkages, which means that their structures are not rigid. For example, Monte Carlo simulations of $C_6$ dynamics indicate that this reagent may adopt conformations such that its cross-linking span between SH1 and SH2 can vary from 6.5 to 12.5 Å. Obviously the flexibility of these reagents complicates any attempt to correlate the cross-linking results with distances (or reagent length). Second, there is no obvious reason for normalizing the rates of cross-linking, which is essentially the modification of SH2 on SH1-modified S1 to SH1 modification rates. Thus conclusions drawn from this work must be called into question.

## SUMMARY

Currently, the cross-linking method has been used to probe several states of the SH1-SH2 helix in the myosin head, which are relevant to the cross-bridge cycle. When actin is bound to S1 or S1.MgADP, the SH1-SH2 helix is stable. As ATP binds to form S1.MgATP (as represented by MgATPγS), the helix becomes very flexible. After hydrolysis, the myosin head reattaches to actin filament and the helix returns to a more rigid conformation, as viewed in the acto-S1.MgADP state. The state yet to be investigated is the post-hydrolysis state, represented by the transition state analogs, MgADP.$AlF_4$- and MgADP.$V_i$. Because of the time needed for the formation of these nucleotide and phosphate analog complexes in S1, the cross-linking method used in this study could not be applied to examine these states. Work is now in progress to devise another method to obtain the cross-linking rates of these important states of the cross-bridge cycle and to construct a map of the SH1-SH2 helix states throughout the cross-bridge cycle. Clearly, to assist in comparing the crystal and solution S1 structure and dynamics, cross-linking experiments on scallop S1 will also have to be done.

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## REFERENCES

Aguirre, R., S. H. Lin, F. Gonsoulin, C. K. Wang, and H. C. Cheung. 1989. Characterization of the ethenoadenosine diphosphate binding site of myosin subfragment 1. Energetics of the equilibrium between two states of nucleotide. S1 and vanadate-induced global conformation changes detected by energy transfer. *Biochemistry.* 28:799–807.

Baker, J. E., I. Brust-Mascher, S. Ramachandran, L. E. LaConte, and D. D. Thomas. 1998. A large and distinct rotation of the myosin light chain domain occurs upon muscle contraction. *Proc. Natl. Acad. Sci. USA.* 95:2944–2949.

Batra, R., M. A. Geeves, and D. J. Manstein. 1999. Kinetic analysis of *Dictyostelium discoideum* myosin motor domain with glycine-to-alanine mutations in the reactive thiol region. *Biochemistry.* 38:6126–6134.

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

Cartoux, L., T. Chen, G. DasGupta, P. B. Chase, M. J. Kushmerick, and E. Reisler. 1992. Antibody and peptide probes of interactions between the SH1-SH2 region of myosin subfragment 1 and actin's N-terminus. *Biochemistry.* 31:10929–10935.

Chaussepied, P., D. Mornet, and R. Kassab. 1986. Nucleotide trapping at the ATPase site of myosin subfragment 1 by a new interthiol crosslinking. *Proc. Natl. Acad. Sci. USA.* 83:2037–2041.

Cooke, R. 1997. Actomyosin interaction in striated muscle. *Physiol. Rev.* 77:671–697.

Dominguez, R., Y. Freyzon, K. M. Trybus, and C. Cohen. 1998. Crystal structure of a vertebrate smooth muscle myosin motor domain and its complex with the essential light chain: visualization of the pre-power stroke state. *Cell.* 94:559–571.

Duke, J., R. Takashi, K. Ue, and M. F. Morales. 1976. Reciprocal reactivities of specific thiols when actin binds to myosin. *Proc. Natl. Acad. Sci. USA.* 73:302–306.

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

Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* 60:375–400.

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

Gulick, A. M., C. B. Bauer, J. B. Thoden, and I. Rayment. 1997. X-ray structures of the MgADP, MgATPγS, and MgAMPPNP complexes of the *Dictyostelium discoideum* myosin motor domain. *Biochemistry.* 36: 11619–11628.

Highsmith, S., and O. Jardetzky. 1980. G-actin binding quenches internal motions in myosin subfragment-1. *FEBS Lett.* 121:55–60.

Hiratsuka, Y., M. Eto, M. Yazawa, and F. Morita. 1998. Reactivities of Cys707 (SH1) in intermediate states of myosin subfragment-1 ATPase. *J. Biochem.* 124:609–614.

Houdusse, A., V. N. Kalabokis, D. Himmel, A. G. Szent-Gyorgyi, and C. Cohen. 1999. Atomic structure of scallop myosin subfragment S1 complexed with MgADP: a new conformation for the myosin head. *Cell.* 97:459–470.

Kameyama, T., and T. Sekine. 1973. Effect of F-actin on the reactivity of a specific sulfhydryl group (S2) in heavy meromyosin. *J. Biochem.* 74:1283–1285.

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

Kliche, W., M. T. Pfannstiel, S. Stoeva, and H. Faulstich. 1999. Thiol-specific cross-linkers of variable length reveal a similar separation of SH1 and SH2 in myosin subfragment 1 in the presence and absence of MgADP. *Biochemistry.* 38:10307–10317.

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

Lehrer, S. S., B. Nagy, and J. Gergely. 1972. The binding of $Cu^{2+}$ to actin without loss of polymerizability: the involvement of the rapidly reacting -SH group. *Arch. Biochem. Biophys.* 150:164–174.

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

Moore, P. B., H. E. Huxley, and D. J. DeRosier. 1970. Three-dimensional reconstruction of F-actin, thin filaments and decorated thin filaments. *J. Mol. Biol.* 50:279–295.

Mornet, D., K. Ue, and M. F. Morales. 1985. Stabilization of a primary loop in myosin subfragment 1 with a fluorescent crosslinker. *Proc. Natl. Acad. Sci. USA.* 82:1658–1662.

Nikolaeva, O. P., V. N. Orlov, I. V. Dedova, V. A. Drachev, and D. I. Levitsky. 1996. Interaction of myosin subfragment 1 with F-actin studied by differential scanning calorimetry. *Biochem. Mol. Biol. Int.* 40: 653–661.

Nitao, L. K., and E. Reisler. 1998. Probing the conformational states of the SH1-SH2 helix in myosin: a cross-linking approach. *Biochemistry.* 37: 16704–16710.

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

Polosukhina, K., and S. Highsmith. 1997. Kinetic investigation of the ligand dependence of rabbit skeletal muscle myosin subfragment 1 Cys-697 and Cys-707 reactivities. *Biochemistry.* 36:11952–11958.

Rayment, I. 1996. The structural basis of the myosin ATPase activity. *J. Biol. Chem.* 271:15850–15853.

Roopnarine, O., A. G. Szent-Györgyi, and D. D. Thomas. 1998. Microsecond rotational dynamics of spin-labeled myosin regulatory light chain induced by relaxation and contraction of scallop muscle. *Biochemistry.* 37:14428–14436.

Schwyter, D., M. Phillips, and E. Reisler. 1989. Subtilisin-cleaved actin: polymerization and interaction with myosin subfragment 1. *Biochemistry.* 28:5889–5895.

Shriver, J. W. 1986. The structure of myosin and its role in energy transduction in muscle. *Biochem. Cell Biol.* 64:265–276.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.

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

Walker, M., H. White, B. Belknap, and J. Trinick. 1994. Electron cryomicroscopy of acto-myosin-S1 during steady-state ATP hydrolysis. *Biophys. J.* 66:1563–1572.

Walker, M., X. Z. Zhang, W. Jiang, J. Trinick, and H. D. White. 1999. Observation of transient disorder during myosin subfragment-1 binding to actin by stopped-flow fluorescence and millisecond time resolution electron cryomicroscopy: evidence that the start of the cross-bridge power stroke in muscle has variable geometry. *Proc. Natl. Acad. Sci. USA.* 96:465–470.

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

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