Binding of SH1−SH2-Modified Myosin Subfragment-1 to Actin

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ABSTRACT: Myosin subfragment-1 (S1) was labeled with NPM in the presence of ATP or with pPDM in the presence of ADP at 0 °C, conditions which favor linking of maleimide groups to both Cys-707 (SH1) and Cys-697 (SH2). Unmodified S1 was removed by sedimentation with a small amount of F-actin, and the modified protein in the supernatant thoroughly dialyzed. The myosin high-salt EDTA and calcium ATPase activities of the isolated modified S1 were close to zero, suggesting nearly complete modification of SH1 and SH2. The binding of control and these modified myosins to actin was measured at 100 mM ionic strength using a co-sedimentation assay. In the presence of high MgATP concentration, control and NPM- and pPDM-reacted S1 all bind weakly to actin, with binding constants K_3 of 4.9, 2.2, and 1.9 $\times 10^4$ M^{-1}, respectively. In the absence of MgATP, the binding constant K_2 of pPDM-reacted S1 remains weak, 4.6 $\times 10^4$ M^{-1},while that of NPM-reacted and control S1 becomes strong, 4.7 and 31 $\times 10^6$ M^{-1}, respectively. The binding constant for ATP to acto-NPM-reacted-S1 is $\sim 2 \times 10^4$ M^{-1}. Our data suggest that the binding of NPM-S1 to F-actin, in contrast to that of pPDM-S1, is ATP sensitive and can be quite strong at very low ATP concentration. They also suggest that while simple alkylation of SH1 and SH2 may be sufficient to inhibit myosin's ability to hydrolyze ATP, actual covalent linkage of SH1 and SH2 may be necessary to inhibit the weakly to strongly binding conformational change.

Force generation by muscle is a result of the interaction of myosin-cross-bridges with actin filaments, accompanied by the hydrolysis of ATP. Although a number of proteins are involved in this process, myosin and actin are the most abundant and presumably the most important. Myosin has two highly reactive sulfhydryls, known as SH1 (Cys-707) and SH2 (Cys-697), that are located in what is likely a critical region of the myosin cross-bridge head near the base of the nucleotide binding pocket, and also near the cleft involved in actin binding (*1*). The ability of actin and myosin to generate force in muscle fibers and to hydrolyze ATP under physiological conditions is exquisitely sensitive to modification of these sulfhydryls. When both SH1 and SH2 are modified, a muscle fiber will not produce force and actomyosin will not hydrolyze ATP (*2*−*5*). Two compounds that react with SH1 and SH2 have been studied, *p*-phenylene dimaleimide (pPDM) and *N*-phenylmaleimide (NPM).

pPDM, a bifunctional reagent which has two reactive maleimide groups in a para configuration across a phenyl ring, has been shown to covalently cross-link the SH1 and SH2 sulfhydryls, with one maleimide linking to SH1 and the other to SH2 (*6*). It was further shown (*4*) that treatment of fibers with pPDM results in inhibition of their ability to produce force. We found (*7*) that the cross-bridges in a pPDM-modified muscle do not bind strongly to the actin filaments, even in rigor solution. Solution study similarly showed that pPDM-reacted myosin subfragment-1, even in the absence of ATP, is similar to S1·ATP and S1·ADP·Pi with regard to binding to actin (*8*).

NPM is a compound similar to pPDM, but is a monofunctional reagent, having but a single reactive maleimide attached to a phenyl ring. When muscle fibers are modified with NPM under relaxing conditions, both SH1 and SH2 again have maleimide groups covalently linked to them (*5*), except in this instance, the maleimide rings are not linked. Each maleimide has its own phenyl ring, a situation which presumably allows more freedom of motion compared to when the maleimides are constrained to the para positions of a shared phenyl ring, as for pPDM. Cross-bridges of fibers reacted with NPM, like those for pPDM-reacted cross-bridges, were reported to be locked in a weakly binding configuration (*7*). A finding possibly at variance with this is that of Xu et al. (*9*) who found that NPM-reacted cross-bridges will go into a strongly binding configuration in magnesium-free rigor solution, even though, as reported previously, not in magnesium-containing rigor solution.

The results of Xu et al. (*9*) could be related to a magnesium effect on myosin or on one of the other important proteins to which magnesium binds. This was the motivation for examining in the current work the binding of purified NPM- and pPDM-reacted myosin subfragment-1 to unregulated F-actin in solution. It is found that pPDM-reacted S1 binds to actin weakly, both in the presence and absence of ATP, while NPM-reacted S1 binds to actin weakly in the presence of ATP, but strongly in its absence. These results, like those of Xu et al. (*9*), do not immediately appear compatible with the interpretation of Barnett et al. (*7*) and suggest that NPM

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and pPDM may not cause identical effects on myosin cross-bridges.

MATERIALS AND METHODS

Reagents. NPM and pPDM were obtained from Aldrich Chemical Co. (Milwaukee, WI) and were dissolved in dimethylformamide to a final concentration of 50 and 10 mM, respectively. Dithiothreitol (DTT) was obtained from ICN Biochemicals (Aurora, OH) and ATP and ADP from Sigma (St. Louis, MO).

Proteins. Rabbit skeletal myosin subfragment-1 was prepared by chymotryptic digestion of myosin as previously described (*10*). Rabbit skeletal muscle G-actin was obtained from Cytoskeleton (Denver, CO). G-actin was polymerized in buffer containing 2.0 mM Tris-HCl (pH 8.0), 0.2 mM $CaCl_2$, 0.5 mM DTT, and 4 mM MgATP. After sedimentation, F-actin was stored in 100 mM KCl, 3 mM $MgCl_2$, 0.0005% NaN_3, and 10 mM imidazole (pH 7.0). The molecular weight for S1 and actin was 120 000 and 42 000, respectively. The extinction coefficients ($A^{1\%}$) at 280 nm were 7.5 cm^{-1} for S1 and 11 cm^{-1} for actin. The concentration of NPM-reacted S1 and pPDM-reacted S1 was determined by Lowry assay (*11*).

NPM Modification of S1. S1 was dialyzed against 165 mM relaxing solution (0.125 M KCl, 4 mM EGTA, 1 mM $MgCl_2$, 4 mM MgATP, and 10 mM imidazole, pH 7.0) at 0 °C. S1 (20 mM) was reacted with 0.2 mM NPM in 165 mM relaxing solution at 0 °C for 120−150 min. The reaction was quenched by adding DTT to a final concentration of 1 mM. About10 mL of the labeled protein solution was dialyzed twice against 1 L of 10 mM imidazole (pH 7.0), 1 mM DTT, and 0.0005% NaN_3 at 0 °C. The protein solution was concentrated to 1.0−1.5 mL. Precipitate formed during preparation was removed by centrifugation. The preparation showed nearly complete loss of EDTA-ATPase and less than 20% of the Ca^{2+}-ATPase activity of S1, suggesting that all of the SH1 and more than 95% of the SH2 sulfhydryls were modified by NPM (*12*).

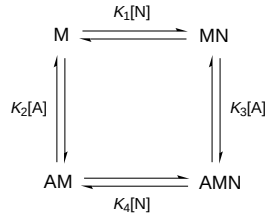
pPDM Modification of S1. S1 was reacted with pPDM at 0 °C as described by Wells and Yount (*13*). Modified S1 was further purified with F-actin (*8*).The final protein had a high-salt EDTA-ATPase activity less than 0.1% and Ca-ATPase activity less than 5% of unmodified S1.

ATPase Assay. The high-salt EDTA and calcium ATPase activities were measured in a buffer containing 10 mM EDTA, 0.6 M KCl, and 50 mM MOPS (pH 7.5), or 10 mM $CaCl_2$, 0.6 M KCl, 50 mM MOPS (pH 7.5). The reaction was initiated (25 °C) by adding 0.1 M ATP to achieve a final concentration of 5 mM, and the production of inorganic phosphate over a 5 min period was measured (*12, 14, 15*).

Binding of S1 to F-Actin: Experimental. F-actin (3 mM) was incubated with the different concentrations of S1 or modified S1 at 25 °C in a solution containing 3 mM EGTA, 1 mM $MgCl_2$, 90 mM KCl, 1 mM DTT, and 10 mM imidazole (pH 7.0). After 10 m, ATP was added to get a final MgATP concentration of 0−1 mM. After co-sedimentation of F-actin and any bound S1, the amount of S1 bound to F-actin and that free in the solution were quantified on a 12% SDS−PAGE gel with a densitometer.

Binding of S1 to F-Actin: Analysis. Assuming simple one-step binding of nucleotide to S1 and of S1 or nucleotide-S1 to actin leads to the following simple scheme for binding (*16*),

Scheme 1



where M is myosin subfragment-1 or modified myosin subfragment-1, A is F-actin, AM is actomyosin, N is nucleotide, which is ATP in this study, and MN and AMN are the nucleotide associated myosin and actomyosin complexes. K_1, K_2, K_3, and K_4 are association constants.

Fitting of Binding Constants. The equations of binding appropriate to Scheme 1 are derived in Appendix 1. In the absence of nucleotide, or at infinite nucleotide, the equations are few in number and yield simple, well-known, analytical solutions. To solve the equations at intermediate values of ATP requires solution of six simultaneous equations as shown in Appendix 1. Fitting either the analytic solutions or the simultaneous equations to the data was done using a C program written by M.S. The program used the standard least-squares procedure based upon Marquardt's compromise (*17*). Copies of the program are available via ftp upon request. All reported values are given as the mean ± 95% confidence limit.

RESULTS

Our first step in understanding the changes produced by different SH1−SH2 alkylating agents was to examine the binding properties of unmodified myosin subfragment-1. This also allows validation of our technique by comparison with data obtained by others. Figure 1a shows the binding of unmodified S1 to F-actin in the absence of MgATP. The averaged data from experiments on three separate preparations are displayed in terms of a plot of concentration of S1 bound versus total S1 concentration. Fitting the equation $K_2 = [AM]/([A][M])$ (in line with the assumption of one-step binding) to the data gives $K_2 = (3.1 \pm 2.4\ 0 \times 10^7$ M^{-1}. Although the accuracy of K_2 may not be as good as suggested by the calculated 95% confidence limit, since the S1concentrations used in the binding experiment were significantly larger than $1/K_2$, it is of interest that the value we obtained is similar to those determined previously with different techniques (*18, 19*). In the presence of 1 mM MgATP (4 preparations, Figure 1b), the binding is several orders of magnitude weaker. Fitting the equation $K_3 = [AMN]/([A][MN])$ to the data gives $K_3 = (4.9 \pm 0.5) \times 10^4$ M^{-1}.

Figure 2 shows the binding of pPDM-reacted S1 to F-actin in the absence of MgATP and in the presence of 1 mM MgATP. A least-squares fit of the data yields binding constants of $K_2 = (4.6 \pm 0.6) \times 10^4$ M^{-1} and $K_3 = (1.9 \pm 0.1) \times 10^4$ M^{-1}. Thus, the binding of pPDM-S1 to actin in the absence of ATP is <3-fold stronger than in its presence. This is consistent with previously reported results that pPDM treatment of myosin in the presence of ADP leads to cross-

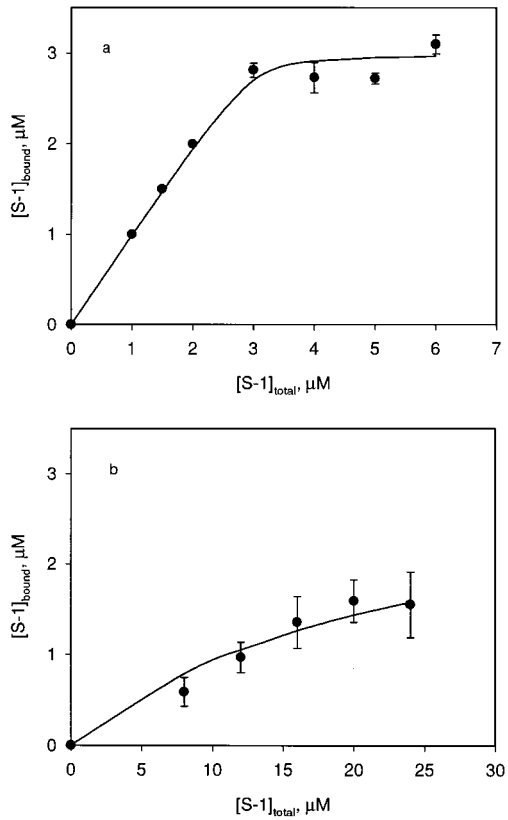


FIGURE 1: The binding of unmodified subfragment-1 to actin. (a) Absence of ATP; (b) 1 mM ATP. Buffer contains 3 mM EGTA, 1 mM $MgCl_2$, 90 mM KCl, 1 mM DTT, and 10 mM imidazole (pH 7.0, 25 °C). Solid curves give best least-squares fit of simple binding equation yielding (a) $K_2 = 3.1 \times 10^7$ M^{-1} and (b) $K_3 = 4.9 \times 10^4$ M^{-1}.

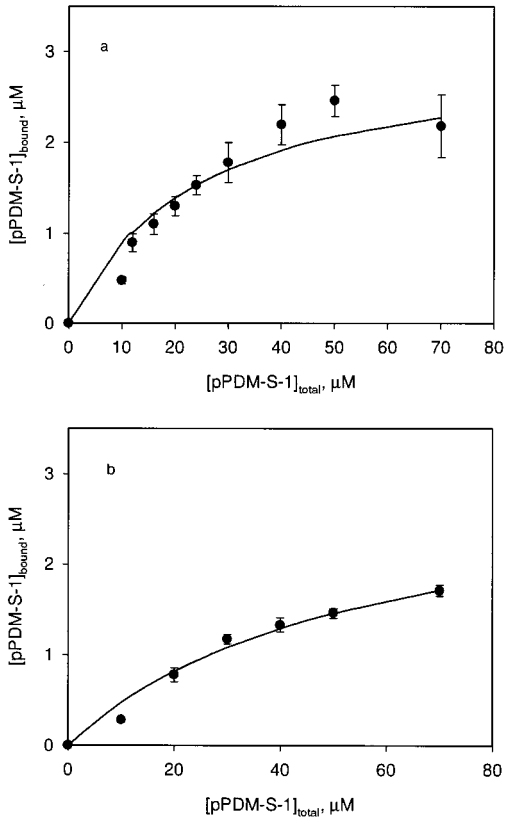


FIGURE 2: The binding of pPDM-modified subfragment-1 to actin. (a) Absence of ATP; (b) 1 mM ATP. Same conditions as Figure 1. Solid curves give best least-squares fit of simple binding equation yielding (a) $K_2 = 4.6 \times 10^4$ M^{-1} and (b) $K_3 = 1.9 \times 10^4$ M^{-1}.

linking of SH1 and SH2, inhibition of the actomyosin ATPase, and locks myosin in a weakly binding conformation (*6, 20, 8*).

Figure 3 shows the binding of NPM-reacted S1 to F-actin in the absence of MgATP and in the presence of 1 mM MgATP. The binding constant of NPM-S1 to F-actin in the presence of 1 mM MgATP is $K_3 = (2.2 \pm 0.2) \times 10^4$ M^{-1}, about the same as for unmodified and pPDM-reacted S1. In the absence of MgATP, the binding constant is $K_2 = (4.7 \pm 0.6) \times 10^6$ M^{-1}, a value most similar to the binding constant of unmodified S1 under rigor conditions. This result, seemingly in conflict with the findings of Barnett et al. (*7*), demonstrates that NPM-S1 binds relatively tightly to F-actin in the absence of ATP and quite weakly in its presence. The data of Figure 3, however, do not provide information about the sensitivity of acto-NPM-S1 to ATP dissociation.

To obtain the sensitivity of acto-NPM-S1 to ATP dissociation, one must determine K_4, the binding constant of MgATP to acto-NPM-S1. Doing this requires binding data at intermediate values of [MgATP]. Figure 4 shows binding curves for MgATP concentrations of 0, 5, 10, 20, 40, 80, 160, and 1000 mM. The best least-squares fit of the model represented by Scheme 1 to the data of Figure 4, obtained as outlined in Appendix 1, is shown by the solid lines. The binding constants determined from the fit are $K_2 = (3.5 \pm 1.9) \times 10^6$ M^{-1}, $K_3 = (3.3 \pm 0.6) \times 10^4$ M^{-1}, and $K_4 = (2.2 \pm 0.8) \times 10^4$ M^{-1}. This shows that acto-NPM-S1 is actually quite sensitive to dissociation by MgATP, the dissociation constant, $1/K_4$, being $\sim$50 μM.

Table 1 summarizes the directly measured association constants, obtained from Figures 1−3, and Table 2 summarizes those derived from Figure 4. A satisfying aspect to the fit of Scheme 1 to the Figure 4 data is that the values determined for K_2 and K_3 from the fit to the data as a whole, are similar to those determined from the 0 mM and 1 mM MgATP data of Figure 3. This suggests, at least, that the data of Figure 4 are consistent, since the value of K_2 should be uniquely determined from the 0 MgATP data, and the value of K_3 should be uniquely determined from a binding curve obtained in the presence of large concentrations of ATP. The finding that the apparent dissociation constant for MgATP binding to acto-NPM-S1 is < 100 μM justifies the use of 1 mM MgATP to determine K_3. A less satisfying aspect of the fit is that the binding data show considerably more sigmoidicity than the best-fit curves.

DISCUSSION

Barnett et al. (*7*) found that the cross-bridges in fibers reacted with NPM or pPDM appear locked in a weakly binding configuration which persists even after ATP is removed from or calcium is added to the bathing media. The pPDM data were consistent with earlier results, suggesting that pPDM treated subfragment-1 was a good analogue of the weakly binding S1·ATP state (*8*). The Barnett et al. (*7*) results suggested that the effect of NPM on the cross-bridges was nearly identical to that of pPDM. This conclusion was questioned by more recent results of Xu et al. (*9*), who found that NPM-reacted cross-bridges will go into a strongly binding configuration when magnesium, in addition to ATP, is removed from the bathing media. Although Barnett et al.

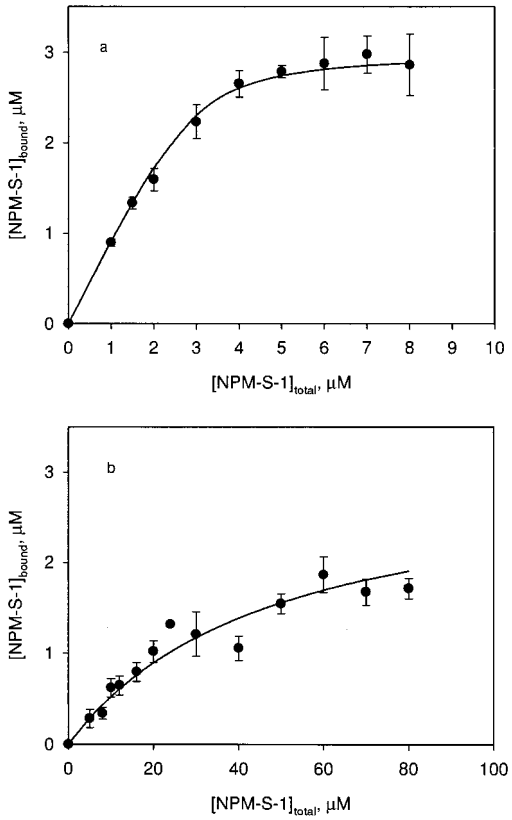


FIGURE 3: The binding of NPM-modified subfragment-1 to actin. (a) absence of ATP; (b) 1 mM ATP. Same conditions as Figures 1 and 2. Solid curves give best least-squares fit of simple binding equation yielding (a) $K_2 = 4.7 \times 10^6$ M^{-1} and (b) $K_3 = 2.2 \times 10^4$ M^{-1}.

always had magnesium in their solutions, it is unclear whether the results of Xu et al. and Barnett et al. are compatible. The components of the solutions in the present experiments were similar to those in Barnett et al., 1992, making comparison easier. Although the pPDM results obtained here seem compatible with the earlier findings of Barnett et al., the NPM results do not.

The present results suggest that NPM and pPDM do not behave identically with regard to their effect on cross-bridges, even though both react, under the treatment conditions of this study, with both SH1 and SH2 (*5*). NPM and pPDM do appear similar with regard to their capability of inhibiting myosin's ability to hydrolyze ATP, both at high salt [confirming the reaction at SH1 and SH2 (*12*)] and also under physiological conditions (unpublished observations). They are again similar in that both pPDM- and NPM-reacted myosin bind weakly to actin in the presence of ATP. However, the two differ dramatically in their affinity for actin in the absence of ATP. In the absence of ATP, the affinity of pPDM-S1 for actin is about 100-fold weaker than the affinity of NPM-S1 for actin and only about 2-fold stronger than the affinity of NPM-S1 or pPDM-S1 for actin in the presence of ATP. This behavior of NPM-S1 is more similar to that of NEM-S1[1] than to pPDM-S1.

[1] Most studies of the effects of NEM on myosin have been on extensively modified myosin. However, Burke et al. (22) followed a milder protocol that linked NEM to just SH1 and SH2. The binding properties of our SH1−SH2-reacted NPM-myosin is very similar to what they found for SH1−SH2-reacted NEM-myosin.

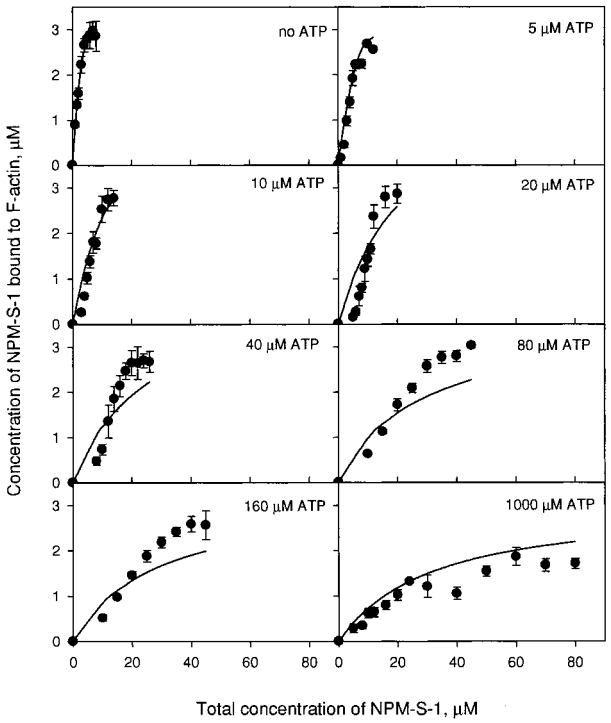


FIGURE 4: The binding of NPM-modified subfragment-1 to actin in the presence of different concentrations of ATP. Same conditions as Figures 1−3. Solid curves show best least-squares fit of Scheme 1 to the data yielding the equilibrium-binding constants given in Table 2.

Table 1: Directly Measured Association Constants (M^{-1})

	unmodified S1	NPM-reacted S1	pPDM-reacted S1
K_2	$(3.1 \pm 2.4) \times 10^7$	$(4.7 \pm 0.6) \times 10^6$	$(4.6 \pm 0.6) \times 10^4$
K_3	$(4.9 \pm 0.5) \times 10^4$	$(2.2 \pm 0.2) \times 10^4$	$(1.9 \pm 0.1) \times 10^4$

Table 2: Association Constants Derived from Figure 4 (M^{-1})

	unmodified S1[a]	NPM-reacted S1[b]
K_1	10^{11}	$(2.3 \pm 1.6) \times 10^6$
K_2	$(3.1 \pm 2.4) \times 10^7$	$(4.7 \pm 0.6) \times 10^6$
K_3	$(4.9 \pm 0.5) \times 10^4$	$(3.3 \pm 0.6) \times 10^4$
K_4	$(1.6 \pm 1.2) \times 10^8$	$(2.2 \pm 0.8) \times 10^4$

[a] For unmodified S1, K_2 and K_3 come from Figure 1 and K_1 from Trentham et al. (*23*). [b] For NPM-reacted S1, K_2, K_3, and K_4 come from Figure 4. Other values calculated from $K_1K_3 = K_2K_4$.

A major structural difference between the pPDM moiety and NPM or NEM moieties is whether the maleimide linked to SH1 is covalently linked to the maleimide linked to SH2. In the case of pPDM, the SH1- and SH2-reacting maleimide groups are linked through the common phenyl ring which joins them. It is thought that the conformational change between the strongly binding and weakly binding conformations involves relative movement of the SH1 and SH2 sulfhydryls (*21*). One reason that pPDM-reacted myosin cannot go into a strongly binding configuration could be that the covalent linkage of SH1 and SH2 (through the maleimide and common phenyl rings) prevents this motion. The results suggest that simple alkylation of SH1 and SH2 is sufficient to inhibit myosin's ability to hydrolyze ATP, but actual covalent linkage of SH1 and SH2 may be necessary to inhibit the weakly to strongly binding conformational change.

As seen from Table 2, NPM modification of subfragment-1 has its major effect on the binding of ATP to myosin's

nucleotide binding pocket, producing a $>10^4$ change in the affinity constant of ATP to S1 or acto-S1 and relatively little effect on the binding of S1 to actin, either in the presence or absence of nucleotide. One way in which this might be consistent with NPM's ability to greatly reduce the ATPase rate would be if the NPM modification reduced the on-rate of ATP binding. This reduction in the on-rate would reduce the affinity constant and presumably the ATPase rate, but still allow the weakly to strongly binding conformational change once ATP finally bound to the binding pocket.

Although the sigmoidal appearance of the experimental data in Figure 4 could signify cooperative binding, a more likely explanation is that it is due to residual ATPase activity of NPM-reacted S1. If NPM-reacted S1 has residual ATPase activity, with increase of concentration of total S1, more ATP would be hydrolyzed during the incubation period and the true ATP concentration would be less than thought, leading to proportionately more binding of S1 to actin. Although the actin-activated ATPase activity of NPM-reacted S1 is only 1/20th that of unmodified S1 (Xie et al., manuscript in preparation), it is still significant enough to account for the sigmoidal appearance of the data in Figure 4. Because of this, the fit of model to the data is not perfect, and the specific value determined for the ATP dissociation constant is likely only approximate. One thing, however, is quite clear. Only a small amount of ATP significantly shifts the binding curves for NPM-reacted S1, binding in the presence of ATP being much weaker than in its absence.

In summary, even though both NPM-reacted and pPDM-reacted subfragment-1 have maleimide groups linked to both SH1 and SH2, the behavior of NPM-reacted S1 is not the same as that of pPDM-reacted S1. Both species have greatly reduced ATPase activities, and both bind to actin weakly in the presence of ATP. However, while pPDM-reacted S1 still binds to actin weakly even in the absence of ATP, NPM-reacted S1 binds to actin strongly. We hypothesize that this inability of pPDM-reacted S1 to go into a strongly binding configuration under conditions where NPM-reacted S1 does may be due to a lessening of the ability of SH2 to move relative to SH1 because of an inflexible covalent linkage between them in the case of pPDM.

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APPENDIX 1

In the absence of ATP, or when the ATP concentration is high enough to saturate all the myosin subfragment-1, Scheme 1 reduces to simple bimolecular binding. In either case, there is just one binding and two mass-conservation equations and the analytical solutions relating the amount of myosin bound as a function of the given total actin and myosin concentrations are well-known and not reproduced here.

When the ATP concentration is intermediate between these two extremes, there is trimolecular binding, and the resulting set of equations is more complex, with three binding and three mass-conservation equations. These are

$$[\mathrm{AM}]/[\mathrm{M}] = K_2[\mathrm{A}] \quad (1)$$

$$[\mathrm{AMN}]/[\mathrm{MN}] = K_3[\mathrm{A}] \quad (2)$$

$$[\mathrm{AMN}]/[\mathrm{AM}] = K_4[\mathrm{N}] \quad (3)$$

$$[\mathrm{N}] + [\mathrm{MN}] + [\mathrm{AMN}] = N_\mathrm{o} \quad (4)$$

$$[\mathrm{A}] + [\mathrm{AM}] + [\mathrm{AMN}] = A_\mathrm{o} \quad (5)$$

$$[\mathrm{M}] + [\mathrm{MN}] + [\mathrm{AM}] + [\mathrm{AMN}] = M_\mathrm{o} \quad (6)$$

where A is actin, M is myosin subfragment-1, N is ATP, and the combined symbols refer to the bound bi- or trimolecular species. The total (known) concentrations of actin, myosin, and ATP are A_o, M_o, and N_o, respectively.

The experimental-binding data are given in terms of the amount of myosin bound (y) as a function of the total myosin (x). In terms of previously defined variables, $x = M_o$, and $y = [\mathrm{AM}] + [\mathrm{AMN}]$. The total actin and total nucleotide in a given experiment are known and fixed.

With K_2, K_3, K_4, A_o, and N_o as known parameters, we have six equations and six unknowns. Equations 1−3 can be used to express [A], [M], and [N] in terms of [AM], [MN], and [AMN]. When these expressions are substituted into eqs 4−6, the equations and unknowns are reduced to three. Equation 4 can be used to express [AMN] in terms of [AM] and [MN]. This can be put into eq 5, which can be used to express [MN] in terms of [AM]. After the expressions for [AMN] and [MN] are put into eq 6, there remains a single, rather complicated, equation containing [AM] only, and, of course, the parameters x, K_2, K_3, K_4, A_o, and N_o. Equation 6 can be solved for [AM] as a function of these parameters, after which eq 5 can be used to solve for [MN], and eq 4 for [AMN]. This yields a solution for the amount of bound myosin, $y = [\mathrm{AM}] + [\mathrm{AMN}]$, and the concentrations of all the intermediates in Scheme 1 can readily be determined.

Derivation of the expression for [AMN] from eq 4 and [MN] from eq 5 was done by hand and using the symbolic language program Mathematica (Wolfram Research, Inc., Champaign, IL) with identical result. Substitution in eq 6 was done using Mathematica alone and resulted in an equation with 81 terms. This equation could not be solved analytically by Mathematica, but we were able to obtain a numerical solution on a Hewlett-Packard 725/50 workstation (Palo Alto, CA) using a successive approximation method encoded in C. Calculation of the solution for the concentrations of all intermediates in Scheme 1 required less than 3 s of elapsed time. Once obtained, the solutions were checked easily by hand in the original (unsubstituted) equations.

With the ability to calculate y as a function of x, K_2, K_3, K_4, A_o, and N_o, it was possible to determine the values of K_2, K_3,and K_4 that gave the best fit of Scheme 1 to the experimental x, y data points. This was done using a

nonlinear least-squares fitting procedure, based upon Marquardt's compromise (*17*) and written in C by M.S. Copies of all programs via email or ftp are available upon request.

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