Interaction of Spin-Labeled and *N*-(Iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic Acid SH_1-Blocked Heavy Meromyosin and Myosin with Actin and Adenosine Triphosphate†

Sally A. Mulhern and Evan Eisenberg*

ABSTRACT: It has previously been demonstrated that when the SH_1 group of heavy meromyosin is modified with *N*-ethylmaleimide there is a marked reduction in the acto-heavy meromyosin ATPase activity, and to account for this reduction in actin activation it was postulated that the rate-limiting transition from the refractory state of heavy meromyosin, which in general does not bind to actin, to the nonrefractory state of heavy meromyosin, which does bind to actin, is much slower with *N*-ethylmaleimide-SH_1-blocked heavy meromyosin than with unmodified heavy meromyosin. In the present study the ATPase activity of heavy meromyosin modified at the SH_1 group with both the spin-label *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)iodoacetamide and the fluorophore *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid was investigated. In addition, the ATPase activity of myosin labeled with the latter reagent was studied. Since these probes have been employed to study the configuration around the active site of myosin and the movement of the myosin cross-bridge in vivo, it is of interest to determine if modification with these reagents has affected important functional properties of the myosin molecule. Our results indicate that, whether or not dithiothreitol is present, the actin activation of heavy meromyosin modified with the fluorophore or spin-label is reduced just as with *N*-ethylmaleimide. Furthermore, near V_{max} most of the modified heavy meromyosin and actin are dissociated, indicating that the rate-limiting transition from the refractory state to the nonrefractory state is reduced just as with *N*-ethylmaleimide-SH_1-blocked heavy meromyosin. This, in turn, suggests that most of the modified HMM still undergoes a cycle of detachment and reattachment from actin as ATP is hydrolyzed. Our results also indicate that at high molar ratios of actin to myosin, blocking the SH_1 group of myosin markedly reduces the actin-activated SH_1-blocked myosin ATPase so that it is about equal to the actin-activated ATPase of the SH_1-blocked HMM. We conclude that probes attached to the SH_1 group of myosin markedly affect the myosin ATPase activity so that, although labeled cross-bridges may still undergo a cycle of interaction with actin in vivo, it is very likely that certain steps in the cycle have markedly altered rate constants.

It is now generally accepted that muscle contraction is driven by myosin and actin filaments sliding past each other with the energy for this process provided by ATP hydrolysis. The myosin molecule is composed of a tail portion, light meromyosin (LMM[1]), and a head portion, heavy meromyosin (HMM). HMM, in turn, contains two nearly identical subunits, subfragment 1 (S-1), each of which has both an actin and an ATP binding site. The myosin molecule itself contains approximately 42 sulfhydryl groups (Huszar & Elzinga, 1971; Barany et al., 1964; Kominz et al., 1954), the majority of which reside in the head portion of the molecule (Lowey et al., 1969). One of these sulfhydryl groups, the SH_1 group, is very reactive and can thus be specifically modified with many different sulfhydryl reagents. This modification results in an activation of the Ca^{2+}-ATPase activity with a concomitant loss in EDTA ATPase activity. Furthermore, when *N*-ethylmaleimide (NEM) is used to block the SH_1 group of HMM, only threefold actin-activation occurs compared to 200-fold activation with unmodified HMM (Silverman et al., 1972). The question as to which step in the acto-HMM ATPase cycle is affected by the modification with NEM was answered by ascertaining the amount of NEM-SH_1-blocked HMM bound to actin when the ATPase was maximally activated by actin (V_{max}). The results indicate that most of the NEM-SH_1-blocked HMM is dissociated from actin near V_{max}. This, in turn, suggests that NEM-SH_1-blocked HMM, like unmodified HMM, exists for most of the cycle of ATP hydrolysis in a refractory state which is unable to bind to actin. To account for the reduction in the actin-activated ATPase of NEM-SH_1-blocked HMM it was postulated that the rate-limiting transition from the refractory to the nonrefractory state is much slower with NEM-SH_1-blocked HMM than with unmodified HMM (Mulhern & Eisenberg, 1976).

Because the SH_1 group is very reactive, it has been selectively modified with various probes to study both the configuration around the active site of myosin and the movement of the myosin cross-bridge in vivo. Two such probes which have been employed for this purpose are the spin-label *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)iodoacetamide (Seidel & Gergeley, 1972) and the fluorophore *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid (1,5-IAEDANS) (Mendelson et al., 1973). Clearly, the interpretation of experiments where these probes are employed depends on whether the probes, themselves, affect important functional properties of the myosin molecule. Therefore, several studies have been performed to determine the effect of these reagents on both the myosin and actomyosin ATPase activity. All of the studies agree that, similar to NEM, both of these reagents activate the Ca^{2+}-ATPase and inhibit the EDTA ATPase of

† From the Section on Cellular Physiology, Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received September 1, 1977; revised manuscript received July 7, 1978.*

[1] Abbreviations used are: HMM, heavy meromyosin; LMM, light meromyosin; S-1, subfragment 1; EDTA, ethylenediaminetetraacetic acid; ATPase, adenosine triphosphatase; NEM, *N*-ethylmaleimide; 1,5-IAEDANS, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid; spin-label, *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)iodoacetamide; DTT, dithiothreitol; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

myosin alone (Seidel, 1973; Takashi et al., 1976). However, the effect of these reagents on the actomyosin ATPase is not as clear. Based on the results with NEM noted above, it might be expected that both 1,5-IAEDANS and the spin-labeled derivative of iodoacetamide would at least to some extent reduce the actomyosin ATPase activity, and indeed both Seidel (1973), using myosin, and Stone (1973), using HMM, found that labeling with the spin-labeled derivative of iodoacetamide reduced the actin-activated ATPase when dithiothreitol (DTT) was *not* added following the labeling reaction. However, Seidel (1973) suggested that this reduction in actin-activated ATPase might be an artifact, since when DTT was added following the labeling reaction, the spin-labeled derivative had no effect on the actomyosin ATPase activity. Similar results were obtained by Mendelson et al. (1975) and Lin & Morales (1977) using 1,5-IAEDANS; treating myosin with DTT after labeling with 1,5-IAEDANS preserved the myosin's full actin-activated ATPase activity.

Since NEM has a marked effect on the acto-HMM ATPase while the spin-labeled derivative of iodoacetamide and 1,5-IAEDANS both appear to have little effect on the actomyosin ATPase, in the present study we investigated the effect of the spin-labeled derivative of iodoacetamide and 1,5-IAEDANS on the acto-HMM ATPase activity. We also investigated the effect of 1,5-IAEDANS on the actomyosin ATPase at high actin concentration. Our results indicate that whether or not DTT is present both spin-labeled and 1,5-IAEDANS-SH_1-blocked HMM show greatly reduced actin-activated ATPase activity. Furthermore, our results show that (using HMM labeled either with the spin-labeled derivative of iodoacetamide or 1,5-IAEDANS) when the actin-activated ATPase is close to V_{max} most of the modified HMM and actin are dissociated. This, in turn, suggests that modification by the spin-labeled derivative of iodoacetamide or 1,5-IAEDANS inhibits the actin-activated ATPase by reducing the rate of the transition from the refractory state to the nonrefractory state just as occurs with NEM-SH_1-blocked HMM. Our results also show that blocking the SH_1 group of myosin with 1,5-IAEDANS reduces the actin-activated ATPase activity of myosin at high actin concentration, to approximately the same level obtained with 1,5-IAEDANS-SH_1-blocked HMM. We conclude that the reduction in actin-activated ATPase caused by sulfhydryl reagents does not depend on the specific sulfhydryl reagent which is used and occurs with both myosin and HMM.

Materials and Methods

Protein Preparation. Rabbit skeletal myosin was prepared by the method of Kielley & Harrington (1960), and HMM was prepared from myosin or SH_1-blocked myosin by the method of Eisenberg & Moos (1968). Actin was prepared by a modified method of Spudich & Watt (1971) (Fraser et al., 1975). For binding experiments in the absence of ATP, free ATP was removed from the F-actin solution by mixing it with a 50% suspension of Dowex 1-X anion-exchange resin (0.1 volume of actin). The mixture was then filtered through a fritted glass Buchner funnel to remove the Dowex resin.

Labeling the SH_1 Group of Myosin. The spin-label used was a generous gift from either Dr. John Seidel at the Boston Biomedical Research Institute or Dr. Colin Chignell at the National Institutes of Health. The spin-label was dissolved in ethanol at a concentration of 20 mg/mL and diluted 100-fold with 40 mM Tris (pH 8) before use. To label the SH_1 group, myosin at 20 mg/mL was incubated with a tenfold molar excess of spin-label, unless otherwise stated. Conditions for the labeling reaction were 0.5 M KCl, 40 mM Tris (pH 8), 0 °C. After 45 min the reaction was stopped by the addition of a 50-fold molar excess of DTT over spin-label or by a tenfold dilution with 2 mM imidazole (pH 7). When the reaction was stopped with 50 mM DTT the protein was then concentrated with ammonium sulfate (50%), whereas when the reaction was stopped by dilution the precipitated protein was first centrifuged and the pellet was then dissolved to the desired concentration in 0.5 M KCl, 2 mM EDTA.

TABLE I: Effect of Labeling the SH_1 Group of Myosin on the ATPase Activity of Myosin.[a]

protein	ATPase act. (s^{-1}) Ca^{2+}	ATPase act. (s^{-1}) EDTA
unmodified myosin	2.5	11
NEM myosin	12	0.5
spin-labeled myosin	24	0.8
1,5-IAEDANS myosin	22	0.9

[a] The conditions are given in the legend to Figure 1. The values for unmodified and NEM-SH_1-blocked myosin were obtained from Silverman et al. (1972).

The fluorophore, 1,5-IAEDANS, was purchased from Aldrich Chemical Co. Generally, a modified method of Takashi et al. (1976) was used to block SH_1 with 1,5-IAEDANS. Myosin at 20 mg/mL was incubated with 1,5-IAEDANS at an 80-fold molar excess of 1,5-IAEDANS to myosin molecules. Conditions for the blocking reaction were 0.6 M KCl, 50 mM Tes (pH 7), 0 °C. After 45 min, the reaction was stopped by the addition of a 60-fold molar excess of DTT, and the myosin was then precipitated by a 20-fold dilution with 2 mM DTT. The myosin was then redissolved at an appropriate concentration in 0.5 M KCl, 2 mM imidazole (pH 7.0), and 2 mM DTT and dialyzed against this same solvent. In all of the subsequent storage and handling of the myosin, including the preparation and storage of HMM, 2 mM DTT was present. Although this method was used to block SH_1 in all experiments where SH_1-blocked myosin was studied and in many experiments where SH_1-blocked HMM was used, no difference was observed in the properties of the SH_1-blocked HMM if ammonium sulfate (50%) precipitation was used instead of dilution or if the HMM was not stored in 2 mM DTT.

Binding and ATPase Measurements. Ultracentrifuge experiments were performed at 15 °C in a Model E analytical ultracentrifuge equipped with a photoelectric scanner as described by Eisenberg et al. (1972a,b) and Mulhern et al. (1975). Each sample was incubated in a 15 °C water bath and transferred into a 12-mm double-sector cell as soon as possible after the initiation of the reaction by addition of HMM. The cells were then placed in a two-hole aluminum rotor which was also maintained at 15 °C. The sample was centrifuged at 30 000 rpm, and scans were taken at 288 nm to minimize absorbance due to ATP.

ATPase rates were measured with a pH stat as previously described (Eisenberg & Moos, 1967; Mulhern et al., 1975). For the calculation of rate constants a molecular weight of 500 000 was used for myosin and a molecular weight of 350 000 was used for HMM.

Results

To study the effect of the spin-labeled derivative of iodoacetamide and 1,5-IAEDANS on the actin-activated HMM ATPase, it is important to label the myosin under conditions where the SH_1 group is specifically labeled. For 1,5-IAEDANS we used the conditions where Takashi et al. (1976) found that the SH_1 group was specifically and completely labeled. As shown in Table I, labeling under these conditions led

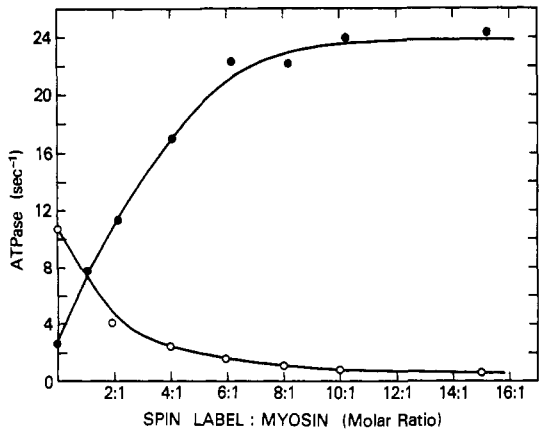

FIGURE 1: Relationship of the Ca^{2+}-ATPase and EDTA ATPase activities to the reaction of myosin with the spin-labeled derivative of iodoacetamide. Conditions for modification are described under Materials and Methods. Conditions for ATPase assay: 0.5 M KCl, 2 mM ATP, 2 mM Tris (pH 7.5), T = 25 °C, and either 1 mM EDTA, 0.16–0.5 μM myosin (○) or 5 mM $CaCl_2$, 0.14–0.64 μM myosin (●).

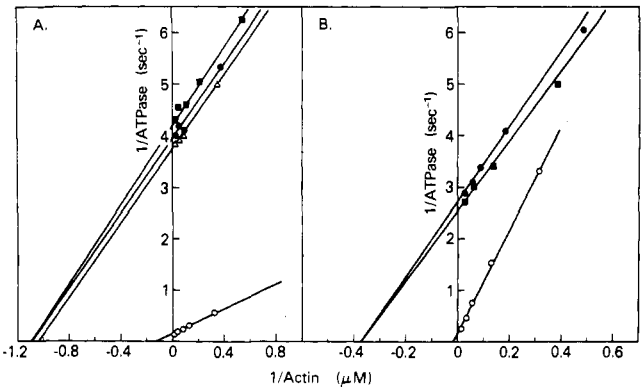

FIGURE 2: Double-reciprocal plots of acto-HMM ATPase vs. added actin concentration using spin-labeled, 1,5-IAEDANS, and unmodified HMM. For each plot the rate of the HMM ATPase in the absence of actin was subtracted from the ATPase rate in the presence of actin (see Table II for values). Conditions were 5.8 μM HMM, 2 mM ATP, 4 mM $MgCl_2$, 3 mM imidazole (pH 7.0), 15 °C. (b) Same as a, except 25 mM KCl was added: (●) 1,5-IAEDANS-SH_1-blocked HMM; (■) spin-labeled-SH_1-blocked HMM (blocking reaction stopped with DTT); (△) spin-labeled-SH_1-blocked HMM (blocking reaction stopped by dilution); (○) unmodified HMM.

to a ninefold increase in the Ca^{2+}-ATPase activity and a 90% inhibition of the EDTA ATPase activity of the HMM. As can be seen, the Ca^{2+}-ATPase and EDTA ATPase activities of the 1,5-IAEDANS-SH_1-blocked HMM were about twice as high respectively as the Ca^{2+}-ATPase and EDTA ATPase activities of the NEM-SH_1-blocked HMM.

To determine the best conditions for labeling with the spin-labeled derivative of iodoacetamide, we labeled at varying ratios of the spin-labeled derivative to myosin. As can be seen in Figure 1, at a 10:1 molar ratio of added spin-labeled derivative to myosin, the Ca^{2+}-ATPase and EDTA ATPase level off. Therefore, we used a 10:1 molar ratio when labeling the myosin with the spin-labeled derivative of iodoacetamide. The fact that the Ca^{2+}-ATPase and EDTA ATPase for the spin-labeled-SH_1-blocked HMM and the IAEDANS-SH_1-blocked HMM are the same (Table I) suggests that under these conditions the blocking with the spin-label is specific as was found by Takashi et al. (1976) for iodoacetamide and 1,5-IAEDANS. However, we did not perform peptide analysis to prove that the labeling was, indeed, specific.

TABLE II: Effect of Labeling the SH_1 Group of Myosin on the Actin-Activated ATPase Activity of HMM.[a]

protein	[KCl] (mM)	ATPase properties −actin ATPase (s^{-1})	+actin V_{max} (s^{-1})	+actin K_{app} (μM)
unmodified HMM	0	0.07	6.27	8
NEM HMM	0	0.05	0.21	0.92
spin-labeled HMM	0	0.17	0.41	0.91
1,5-IAEDANS HMM	0	0.17	0.42	0.99
unmodified HMM	25	0.06	5.51	66
NEM HMM	25	0.06	0.28	2.2
spin-labeled HMM	25	0.25	0.63	2.8
1,5-IAEDANS HMM	25	0.21	0.61	2.8

[a] The conditions are given in the legend to Figure 2. Both the V_{max} and K_{app} were determined from the double-reciprocal plots given in Figures 2a and 2b, except, unlike the values given in the double-reciprocal plots, the ATPase of the HMM alone was *not* subtracted from the maximum actin-activated ATPase rate. The values for NEM HMM were obtained from Mulhern and Eisenberg (1976).

We next investigated the actin activation of HMM made from myosin modified either with 1,5-IAEDANS or the spin-labeled derivative of iodoacetamide. The solid circles in Figure 2A show a double-reciprocal plot of the ATPase activity of 1,5-IAEDANS-SH_1-blocked HMM vs. actin concentration in the absence of KCl, and the solid squares show a similar plot with spin-labeled-SH_1-blocked HMM. For comparison, the open circles in Figure 2A show a double-reciprocal plot using unmodified HMM. Figure 2B shows similar data at 25 mM KCl. As can be seen, both at 0 and 25 mM KCl the actin-activated ATPase activities of the 1,5-IAEDANS-SH_1-blocked HMM and the spin-labeled-SH_1-blocked HMM are almost identical. Both are much lower than the actin-activated ATPase activity of the unmodified HMM.

In all of these experiments, the reaction of myosin with 1,5-IAEDANS or the spin-labeled derivative of iodoacetamide was stopped by the addition of a 50- or 60-fold molar excess of DTT over label (see Materials and Methods). Nevertheless, since the nitroxide radical of the spin-labeled derivative of iodoacetamide reacts with DTT with a resultant loss in ESR signal (Seidel, 1973), we wished to test if the spin-label in the absence of DTT affected the ATPase activity of HMM. Consequently, the spin-labeled-SH_1-blocked myosin was prepared as described above, but the reaction was stopped by tenfold dilution of the sample rather than by addition of a 50-fold molar excess of DTT. The open triangles in Figure 2A show the results. As can be seen, the method used for stopping the reaction had no effect on the actin activation of the spin-labeled-SH_1-blocked HMM ATPase activity.

Table II summarizes the effect of the various sulfhydryl reagents on the Mg-ATPase activity of HMM both in the presence and absence of actin. As can be seen, in the absence of actin at 15 °C, NEM has little effect on the Mg-ATPase activity, whereas both the spin-labeled derivative of iodoacetamide and 1,5-IAEDANS increase the Mg-ATPase activity about three- to fourfold. On the other hand, the maximum actin-activated ATPase activities of the SH_1-blocked HMM species are much reduced compared to that of unmodified HMM, no matter which blocking agent is used. With both spin-labeled- and 1,5-IAEDANS-SH_1-blocked HMM, V_{max} is about twice what it is with NEM, but it is still only about one-tenth of the V_{max} obtained with unmodified HMM. All three blocking agents also had a similar effect on K_{app}, reducing it to about one-tenth the value obtained with un-

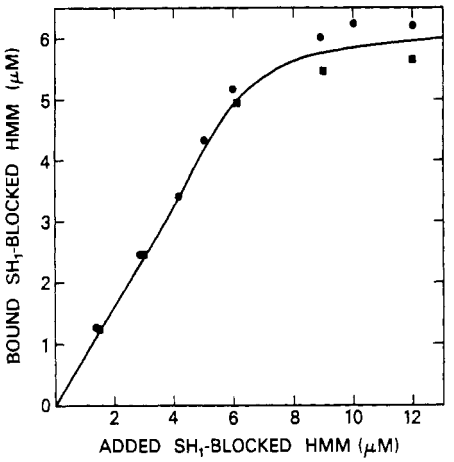

FIGURE 3: Binding of SH_1-blocked HMM to F-actin in the absence of ATP. The analytical ultracentrifuge was employed to measure the binding as described under Materials and Methods. Conditions were 0.1 M KCl, 1 mM $MgCl_2$, 10 mM imidazole (pH 7.0), 5 mM P_i, and 10 μM actin, 15 °C; (●) spin-labeled-SH_1-blocked HMM, (■) 1,5-IAEDANS SH_1-blocked HMM.

modified HMM in the absence of KCl. It is also interesting to note that with all three types of SH_1-blocked HMM, raising the KCl concentration increases K_{app} much less than with unmodified HMM.

We next determined whether, as with NEM-SH_1-blocked HMM, a large fraction of the spin-labeled or 1,5-IAEDANS-SH_1-blocked HMM was dissociated from actin when the actin-activated ATPase was close to its maximal value (V_{max}). As previously, we used the analytical ultracentrifuge to determine the amount of SH_1-blocked HMM bound to actin both in the presence and absence of ATP. Control ultracentrifuge experiments on the binding of spin-labeled and 1,5-IAEDANS-SH_1-blocked HMM to actin in the absence of nucleotide are shown in Figure 3. As can be seen, both the spin-labeled and 1,5-IAEDANS-SH_1-blocked HMM bind to actin quite tightly with saturation occurring at a ratio of 2 mol of actin monomer per mol of SH_1-blocked HMM. Identical results were previously obtained for unmodified (Eisenberg et al., 1972), NEM-SH_1-blocked (Mulhern & Eisenberg, 1976), and spin-labeled HMM (Seidel, 1973) and also for 1,5-IAEDANS-SH_1-blocked S-1 and HMM (Highsmith et al., 1976; Highsmith, 1978), indicating that modification of the SH_1 group has no obvious effect on the interaction of myosin with actin in the absence of ATP.

Next, binding experiments were performed in the analytical ultracentrifuge in the presence of ATP. As shown in Table III, these experiments were performed in both 0 and 25 mM KCl at an actin concentration where the actin-activated ATPase activity is about 95% of V_{max} (see Figure 2). At this actin concentration, in the absence of KCl, approximately 40% of the spin-labeled or 1,5-IAEDANS-SH_1-blocked HMM is bound to actin, while at 25 mM KCl none of the SH_1-blocked HMM binds to actin when the ATPase is very close to V_{max}. These results are identical to the results we previously obtained with NEM-SH_1-blocked HMM and are very similar to the results obtained by Mendelson et al. (1975) using IAEDANS-SH_1-blocked HMM.

The data thus far presented in this paper suggest that whichever SH_1-blocking reagent is employed, it has a major effect on the properties of the actin-activated HMM ATPase. The question which then arises is whether these reagents have any effect on the actomyosin ATPase. As we pointed out in the introductory statement, both Seidel (1973), using the spin-

TABLE III: Amount of Spin-Labeled- and 1,5-IAEDANS-SH_1-Blocked HMM Unbound to Actin during ATP Hydrolysis.[a]

protein	[KCl] (mM)	% V_{max}	% free HMM
spin-labeled HMM	25	95	100
spin-labeled HMM	0	97	57
1,5-IAEDANS HMM	25	94	97
1,5-IAEDANS HMM	0	97	52

[a] The percent V_{max} was calculated from the appropriate double-reciprocal plots shown in Figure 2. The binding studies were performed in the analytical ultracentrifuge as described under Materials and Methods. Conditions: 3 mM $MgCl_2$, 3 mM ATP, 3 mM imidazole (pH 7.0), 2 mM P_i, 5.8 μM SH_1-blocked HMM, 45 μM F-actin, 15 °C.

labeled derivative of iodoacetamide, and Mendelson et al. (1975) and Lin & Morales (1977), using 1,5-IAEDANS, found that labeling the SH_1-group had very little effect on the actomyosin ATPase. However, in these studies from the laboratories of Seidel and Morales only about tenfold actin-activation of the unmodified myosin ATPase was observed. In fact, this is just about the same level of ATPase activity which would be expected with SH_1-blocked myosin if its actin-activated ATPase was the same as the maximum actin-activated ATPase of SH_1-blocked HMM. This is because, in the absence of actin, the Mg-ATPase of SH_1-blocked myosin is already about fourfold higher than the ATPase of unmodified myosin. If actin then increases the ATPase rate of the SH_1-blocked myosin another two- to threefold, the actin-activated ATPase rate of the SH_1-blocked myosin will be just about ten times faster than the ATPase rate of unmodified myosin. Therefore, to determine if blocking SH_1 has an effect on the myosin ATPase, it is necessary to work under a condition where considerably more than tenfold actin activation of the unmodified myosin ATPase is observed.

Of course, measurement of the actomyosin ATPase is somewhat qualitative, since two sets of filaments (actin and myosin) are interacting with each other. Nevertheless, at high actin concentration and relatively low salt concentration, Schliselfeld (1976) found that the actomyosin ATPase can approach values one-fourth to one-half of the V_{max} of the acto-HMM ATPase, which should be quite sufficient to determine whether blocking the SH_1-group affects the actomyosin ATPase.

In the present study, we worked at 25 mM KCl with final protein concentrations of 1 μM myosin and 45 μM actin. The myosin and actin were premixed at high salt concentration and then gradually diluted with stirring. The reaction was then initiated by adding ATP. The results are shown in Table IV for SH_1-blocked and unmodified myosin and HMM. As can be seen, under this condition, the initial actin-activated ATPase of the unmodified myosin is even higher than the actin-activated HMM ATPase under this condition, which is only about one-half V_{max} (see Table II). On the other hand, the initial actin-activated ATPase of the SH_1-blocked myosin is much lower than the actin-activated ATPase of the unmodified myosin and is about equal (80%) to the actin-activated ATPase of the SH_1-blocked HMM. These data strongly suggest that blocking the SH_1 group of myosin does reduce the actin-activated myosin ATPase to about the same level as the actin-activated ATPase of the SH_1-blocked HMM; i.e., approximately threefold actin-activation of the SH_1-blocked myosin occurs so that the actin-activated ATPase of the SH_1-blocked myosin is about ten times higher than the ATPase of unmodified myosin.

TABLE IV: Effect of Labeling the SH_1 Group of Myosin with 1,5-IAEDANS on the Initial Actin-Activated ATPase Activity of Myosin and HMM.[a]

protein	initial ATPase (s^{-1})
unmodified myosin	4.3 (3.7)
1,5-IAEDANS myosin	0.52
1,5-IAEDANS HMM	0.64
unmodified HMM	2.0

[a] Conditions: 2 mM ATP, 4 mM $MgCl_2$, 2 mM imidazole (pH 7.0), 25 mM KCl, 45 μM actin, 1.04 μM unmodified or 1,5-IAEDANS myosin or HMM, 15 °C. The reactions were started on the pH stat by adding 25 mM Mg-ATP (pH 7.0) to a final concentration of 2 mM. The ATPase rates given are averaged for the first minute of the reaction. However, all of the ATPase rates were linear with time except for the rate with unmodified myosin. Here the rate given in parentheses represents the ATPase rate averaged over the first 2 min of the reaction.

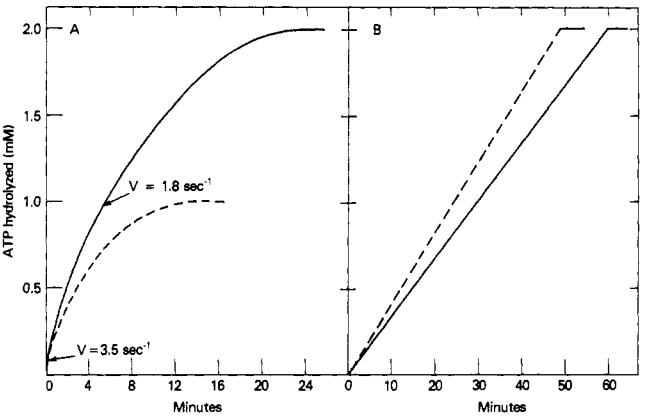

FIGURE 4: Time course of actin-activated ATPase of unmodified and 1,5-IAEDANS-SH_1-blocked myosin and HMM. Conditions were the same as given in Table IV, except as noted below. Figure 4A, solid line: unmodified myosin was present and the reaction was started by making the reaction mixture 2 mM in Mg-ATP. Dashed line: unmodified myosin, 1 mM ADP, and 1 mM P_i were present initially and the reaction was started by making the reaction mixture 1 mM in Mg-ATP. The lower arrow in Figure 4A gives the initial ATPase rate (initial slope) of the dashed line, while the upper arrow gives the ATPase rate (slope) of the solid line when 1 mM ATP has been hydrolyzed. Figure 4B, solid line: IAEDANS-SH_1-blocked myosin present. Dashed line: IAEDANS-SH_1-blocked HMM present.

Figure 4 shows another major difference between unmodified and SH_1-blocked myosin under these conditions. As shown in Figure 4A, the actin-activated ATPase of the unmodified myosin is nonlinear with time, decreasing rapidly as the reaction proceeds. This is not mainly due to inhibition by ADP and P_i. As shown by the dashed curve in Figure 4A, the initial velocity with 1 mM ATP, 1 mM ADP, and 1 mM P_i present is about twice the rate observed at the midpoint of the reaction when 2 mM ATP is added initially. The time-dependent decrease in ATPase may be related to myosin filament formation and superprecipitation of the actomyosin, but at this high molar ratio of actin to myosin, although the solution is turbid, no gross changes could be observed.

As shown in Figure 4B, in contrast to the nonlinear actin-activated ATPase of the unmodified myosin, the actin-activated ATPase of the SH_1-blocked myosin is linear with time and almost identical to the acto-HMM ATPase. Grossly, at least, both before and after ATP addition, the acto-SH_1-blocked myosin solution looked equally turbid to the acto-unmodified myosin solution. The observation that the actin-activated SH_1-blocked myosin ATPase and SH_1-blocked HMM ATPase are so similar raises the interesting question as to whether nonspecific steric factors are indeed responsible for both the frequent observation that under many conditions the actomyosin ATPase is lower than the acto-HMM ATPase (Eisenberg & Moos, 1968; Schliselfeld, 1976) and for the observation in this paper that the actomyosin ATPase is nonlinear with time. In any case, it is clear from the data in Figure 4 and Table IV that blocking SH_1 has major effects on the actomyosin as well as the acto-HMM ATPase.

Discussion

The experiments presented in this paper suggest that both 1,5-IAEDANS and the spin-labeled derivative of iodoacetamide markedly reduce the actin-activated ATPase when they block the SH_1 group of HMM. In fact, the effect of these SH reagents is quite similar to the effect of NEM which we previously observed. As shown in Table II, all three SH reagents decrease the maximum actin-activated HMM ATPase (V_{max}) to about one-tenth the value observed with unmodified HMM and also markedly decrease K_{app}. These effects of the spin-labeled derivative of iodoacetamide or 1,5-IAEDANS are independent of whether or not DTT is used to terminate the blocking reaction.

These results suggest that the effect of blocking the SH_1 group of HMM appears to be relatively independent of the sulfhydryl reagent used. This is of interest because if the SH_1 group were actually at the site where actin or ATP bound one might expect the size of the sulfhydryl reagent to have a major effect on its action. For example, in studies by Wagner and Yount (1975) the inhibition of actin binding, caused by labeling the alkali light chains of myosin with a purine disulfide analogue of ATP, was markedly reduced when the attached analogue was replaced by the smaller thiocyanate group. With the SH reagents used in this paper, no such effect is observed.

There are some minor differences in the ATPase activity depending on which sulfhydryl reagent is used. The results with the spin-labeled derivative of iodoacetamide and 1,5-IAEDANS are essentially identical. With NEM, however, all of the ATPase activities are somewhat lower. The Ca^{2+}-, EDTA, and actin-activated ATPase activities are about one-half the values obtained when the spin-labeled derivative of iodoacetamide or 1,5-IAEDANS is used, whereas the Mg-ATPase in the absence of actin is one-third to one-fourth as great. These differences may be due to a specific effect of NEM on the SH_1 group to the spin-labeled derivative of iodoacetamide or 1,5-IAEDANS. However, it is also possible that NEM does not label the myosin as specifically as the other two sulfhydryl reagents. In fact, there is evidence that, with NEM, minor labeling of other SH groups in the myosin molecule occurs and this might be the cause of the somewhat lower ATPase activity observed with NEM-SH_1-blocked HMM (Sekine et al., 1962).

The data presented in this paper also show that not only does blocking the SH_1 group of HMM markedly reduce the acto-HMM ATPase but, in addition, blocking the SH_1 group of myosin markedly reduces the actomyosin ATPase. At first, it might appear that these data are in disagreement with the results reported by Seidel (1973) using spin-labeled SH_1-blocked myosin and with the results of Mendelson et al. (1975) and Lin & Morales (1977) using 1,5-IAEDANS-SH_1-blocked myosin. In these studies, blocking the SH_1 group had almost no effect on the actin-activated ATPase activity of myosin. However, in these studies the actin-activated SH_1-blocked myosin ATPase was only about tenfold higher than the ATPase of the unmodified myosin alone. Thus, the actin-activated ATPase activity of the SH_1-blocked myosin observed by these

authors is not unlike the actin-activated ATPase activity we observed with SH_1-blocked myosin or HMM as shown in Table IV. The major difference between our results and these previous data is that, by working at relatively high actin concentration, we have obtained much higher levels for the actin-activated ATPase of the unmodified myosin. Therefore, blocking the SH_1 group markedly reduced the actin-activated ATPase in our experiments, while in previous experiments it appeared as if blocking the SH_1 group had little effect.

The data presented in this paper show that blocking the SH_1 group of myosin not only quantitatively reduces the actomyosin ATPase but it has a qualitative effect as well. The actin-activated ATPase of the SH_1-blocked myosin was linear with time, while the actin-activated ATPase of the unmodified myosin was consistently observed to be nonlinear with time, decreasing markedly as the ATP was hydrolyzed. Our results show that this decrease in ATPase is not mainly due to the production of ADP and P_i but rather may be related to some structural change which occurs in the actomyosin precipitate. Presumably then, this structural change, possibly involving myosin filament formation, reduces the actomyosin ATPase so that it becomes much lower than the acto-HMM ATPase measured under identical conditions. It has previously been observed that under many conditions the actomyosin ATPase is lower than the V_{max} of the acto-HMM ATPase, and it was considered possible that nonspecific steric inhibition occurring when the actin and myosin filaments interact might be responsible for this effect (Eisenberg & Moos, 1968; Schliselfeld, 1976). However, our finding that the actin-activated ATPase of the SH_1-blocked myosin is linear with time and almost identical to the actin-activated ATPase of the SH_1-blocked HMM raises a question as to whether nonspecific steric inhibition is indeed responsible for the differences observed between unmodified myosin and HMM in their interaction with actin. An alternative explanation for these differences may be the suggestion of several models of cross-bridge action that the detachment rate of the cross-bridge from actin may be much reduced if the cross-bridge is not free to rotate to its position of minimum free energy or zero force exertion, i.e., rotate to about a 45° angle (Huxley, 1957; Eisenberg & Hill, 1978). It is possible that in an actomyosin suspension, when, after a time, myosin filaments form and interact with the actin filaments, a detachment step of this type might become rate limiting, thereby reducing the actomyosin ATPase rate. On the other hand, with SH_1-blocked myosin, the transition from the refractory to the nonrefractory state may be so slow that it remains the major rate-limiting step even when myosin filaments form and the rate of cross-bridge detachment becomes slow.

Of course this explanation is completely speculative and there are many alternative explanations which might explain the differences observed in the actin activation of unmodified and SH_1-blocked myosin. Nevertheless, our data do strongly indicate that blocking the SH_1 group reduces the actin-activated myosin ATPase to about the same value as the V_{max} of the actin-activated SH_1-blocked HMM ATPase. Thus, our data provides no evidence that a situation can occur where the actomyosin ATPase is *higher* than the V_{max} of the acto-HMM ATPase determined under identical conditions. Clearly, with unmodified myosin the actomyosin ATPase can be slower than the V_{max} of the acto-HMM ATPase, but our data does not support models where the rate-limiting step which determines the V_{max} of the acto-HMM ATPase can be bypassed in the actomyosin system.

The results presented in this paper on the binding of spin-labeled or 1,5-IAEDANS-SH_1-blocked HMM to actin confirm what we previously found with NEM-SH_1-blocked HMM: a large fraction of the SH_1-blocked HMM is dissociated from actin when the ATPase is close to V_{max}. At 25 mM KCl, there is almost no binding of the SH_1-blocked HMM to actin when the ATPase is 94% of V_{max}, while in the absence of KCl 40 to 50% of the HMM is bound. It is of interest that this latter value is the same for unmodified HMM and all of the SH_1-blocked HMM species, no matter which blocking agent is used. As we discussed in a previous paper, the nature of this binding remains unclear (Mulhern & Eisenberg, 1976), although very recent data from our laboratory (Stein et al., unpublished data) suggest that at very high actin concentration and very low salt concentration there is incomplete dissociation of the acto-S-1 complex upon binding ATP. Nevertheless, it appears that a large fraction of the HMM heads are dissociated from actin when the ATPase is close to V_{max}. Therefore, it is quite likely that no matter which sulfhydryl reagent is used most of the SH_1-blocked HMM undergoes a cycle of detachment from, and reattachment to, actin each time an ATP is hydrolyzed, and in this cycle the major rate-limiting step is the transition from the refractory to the nonrefractory state. Furthermore, since the actin-activated ATPase of SH_1-blocked HMM is much slower than the actin-activated ATPase of unmodified HMM, the rate-limiting transition from the refractory to the nonrefractory state must be slower for SH_1-blocked HMM than for unmodified HMM. This, in turn, might be related to the observation that the rate of rigor tension development is slower for skinned fibers labeled with 1,5-IAEDANS than for untreated fibers (Borejdo & Putnam, 1977), but, of course, much more work will be necessary to relate our in vitro findings to studies on fibers where the cross-bridges are labeled with 1,5-IAEDANS.

In this regard, it should be emphasized that, although our data strongly indicate that labeling with 1,5-IAEDANS affects the properties of the myosin molecule, our results also suggest that it is not unlikely that the modified myosin still undergoes a cycle of attachment and detachment from actin when ATP is hydrolyzed in vivo. Furthermore, Borejdo & Putnam (1977) have found that muscle fibers with labeled cross-bridges can exert considerable force. Therefore, although blocking the SH_1 group of myosin undoubtedly affects certain rate constants in the cycle of cross-bridge action, it is very unlikely that it irreversibly denatures the cross-bridge. This, in turn, means that studying the properties of fibers with labeled cross-bridges is of great interest both in understanding how cross-bridges work and in understanding how changes in the ATPase cycle in vitro might affect the action of cross-bridges in vivo.

Acknowledgment

We thank Mr. Louis Dobkin for excellent technical assistance during the course of this project.

References

Barany, M., Gaetjens, E., Barany, K., & Karp, E. (1964) *Arch. Biochem. Biophys. 106,* 280.

Borejdo, J., & Putnam, S. (1977) *Biochim. Biophys. Acta 459,* 578.

Eisenberg, E., & Hill, T. L. (1978) *Prog. Biophys. Mol. Biol. 33,* 55.

Eisenberg, E., & Moos, C. (1967) *J. Biol. Chem. 242,* 2945.

Eisenberg, E., & Moos, C. (1968) *Biochemistry 7,* 1486.

Eisenberg, E., Dobkin, L., & Kielley, W. W. (1972a) *Biochemistry 11,* 4657.

Eisenberg, E., Dobkin, L., & Kielley, W. W. (1972b) *Proc. Natl. Acad. Sci. U.S.A. 69,* 667.

Fraser, A. B., Eisenberg, E., Kielley, W. W., & Carlson, F. C.

(1975) *Biochemistry 14,* 2207.
Highsmith, S. (1978) *Biochemistry 17,* 22.
Highsmith, S., Mendelson, R. A., & Morales, M. F. (1976) *Proc. Natl. Acad. Sci. U.S.A. 71,* 133.
Huszar, G., & Elzinga, M. (1971) *Biochemistry 10,* 229.
Huxley, A. F. (1957) *Prog. Biophys. Biophys. Chem. 7,* 255.
Kielley, W. W., & Harrington, W. F. (1960) *Biochim. Biophys. Acta 41,* 401.
Kominz, D. R., Hough, A., Symonds, P., & Laki, K. (1954) *Arch. Biochem. Biophys. 50,* 148.
Lin, T. I., & Morales, M. F. (1977) *Anal. Biochem. 77,* 10.
Lowey, S., Slayter, H. S., Weeds, A. G., & Baker, H. (1969) *J. Mol. Biol. 42,* 1.
Mendelson, R. A., Morales, M. F., & Botts, J. (1973) *Biochemistry 12,* 2250.
Mendelson, R., Putnam, S., & Morales, M. (1975) *J. Supramol. Struct. 3,* 162.
Mulhern, S. A., & Eisenberg, E. (1976) *Biochemistry 15,* 5702.
Mulhern, S. A., Eisenberg, E., & Kielley, W. W. (1975) *Biochemistry 14,* 3863.
Nihei, T., Mendelson, R. A., & Botts, J. (1974) *Biophys. J. 14,* 236.
Schliselfeld, L. H. (1976) *Biochim. Biophys. Acta 445,* 234.
Seidel, J. C. (1973) *Arch. Biochem. Biophys. 157,* 588.
Seidel, J. C., & Gergeley, J. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 187.
Sekine, T., & Kielley, W. W. (1964) *Biochim. Biophys. Acta 81,* 336.
Sekine, T., Barnett, L. M., & Kielley, W. W. (1962) *J. Biol. Chem. 237,* 2769.
Silverman, R., Eisenberg, E., & Kielley, W. W. (1972) *Nature (London) 240,* 207.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246,* 4866.
Stone, D. B. (1973) *Biochemistry 12,* 3672.
Takashi, R., Duke, J. A., Ue, K., & Morales, M. F. (1976) *Arch. Biochem. Biophys. 175,* 279.
Wagner, P. D., & Yount, R. G. (1975) *Biochemistry 14,* 5156.

Saccharide Binding to Transition Metal Ion Free Concanavalin A†

Douglas J. Christie, Gerald M. Alter, and James A. Magnuson*

ABSTRACT: Saccharide binding has been observed with demetallized concanavalin A in the presence of Ca^{2+} only, using the fluorescent sugar 4-methylumbelliferyl α-D-mannopyranoside. At pH 7.2 both the nicked and intact forms of concanavalin A bound 4-methylumbelliferyl α-D-mannopyranoside with similar affinities. Competitive binding with methyl α-D-mannopyranoside was demonstrated. The association constants at 5 °C were $9.6 \pm 0.6 \times 10^4$ M^{-1} for 4-methylumbelliferyl α-D-mannopyranoside and $1.1 \pm 0.3 \times 10^4$ M^{-1} for methyl α-D-mannopyranoside. 4-Methylumbelliferyl α-D-mannopyranoside binding was also observed if demetallized concanavalin A was remetallized with less than stoichiometric amounts of Ca^{2+}. The association constants with low Ca^{2+} concentrations were similar to those determined with saturating Ca^{2+}. With less than stoichiometric levels of Ca^{2+}, the number of sugar molecules bound per protein subunit was a reflection of the fraction of activated lectin subunits. These results show that saccharide binding activity of concanavalin A does not require a transition metal ion at pH 7.2; only Ca^{2+} is required. At pH values near 5, where most previous studies have been carried out, both a transition metal ion and Ca^{2+} are necessary.

Concanavalin A[1] (Con A), a protein isolated from jack bean (*Canavalia ensiformis*) (Sumner & Howell, 1936), is the most extensively studied lectin. Properties associated with Con A include stimulation of a mitogenic response in lymphocytes (Wecksler et al., 1968; Powell & Leon, 1970; Beckert & Sharkey, 1970) and differential agglutination of malignant and normal cells (Inbar & Sachs, 1969a,b). The induction of these activities is primarily due to the ability of Con A to bind specific carbohydrate moieties on cell surfaces (Lis & Sharon, 1973). Carbohydrates having the D-arabinopyranoside configuration at C-3, C-4, and C-6 positions possess the minimum structural characteristics for binding to Con A (Goldstein et al., 1965, 1973).

Con A exists in two pH-dependent forms each composed of identical subunits. The subunit molecular weight is 25 500 (Wang et al., 1971; Edmundson et al., 1971). The form below pH 5.5 is predominantly dimeric and that found near physiological pH is mostly tetrameric (Kalb & Lustig, 1968; McKenzie et al., 1972). Each subunit possesses one specific carbohydrate binding site (Kalb & Levitzki, 1968; Becker et al., 1975). Con A also possesses a naturally occurring cleavage site (nicked form) in some of the protein molecules (Wang et al., 1971).

Considerable work has been undertaken to investigate the nature of saccharide binding and divalent metal ion interaction

† From the Program in Biochemistry and Biophysics, Department of Chemistry, Washington State University, Pullman, Washington 99164. *Received May 9, 1978.* This research was supported by United States Public Health Service Grant CA 14496. A preliminary report on this work has been presented at the 62nd Annual Meeting of the American Society of Biological Chemists, Atlanta, Ga., June 1978.

[1] Abbreviations used: Con A, concanavalin A; Con A_{int}, intact polypeptide chains of concanavalin A; Mops, morpholinopropanesulfonate; Con A_D, demetallized concanavalin A; Ca^{2+}-Con A (Ca^{2+}-Con A_{int}), demetallized concanavalin A remetallized with Ca^{2+} only; Ca^{2+}-Mn^{2+}-Con A (Ca^{2+}-Mn^{2+}-Con A_{int}), demetallized concanavalin A remetallized with Mn^{2+} and Ca^{2+}; MUM, 4-methylumbelliferyl α-D-manopyranoside; MUG, 4-methylumbelliferyl α-D-glucopyranoside; α-MM, methyl α-D-mannopyranoside; α-MG, methyl α-D-glucopyranoside; $[Ca^{2+}]/[Con\ A]$, total molar Ca^{2+} concentration to the total molar Con A (Con A_{int}) subunit concentration.

0006-2960/78/0417-4425$01.00/0