change of dissociation between bulk and surface amounts to 2.01 kcal/mol at 20 °C.

According to classical conceptions, protein adsorbed at the aqueous surface is unfolded. The results presented above, however, strongly suggest that hemoglobin(III) monolayers consist of globular molecules. From the unexpectedly close similarity of the numerical values of the dissociation constant for the surface and the bulk phase, it appears that the specific interactions governing the dissociation equilibrium and, hence, the overall native conformation are essentially retained. This is even more evident from the similarity of the pH dependence of the constant, indicating that the number of protons bound upon dissociation is the same in either phase.

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Further Studies on the Interaction of Actin with Heavy Meromyosin and Subfragment 1 in the Presence of ATP†

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ABSTRACT: It has been postulated that, during the hydrolysis of ATP, both normal and SH_1-blocked heavy meromyosin undergo a rate-limiting transition from a refractory state which cannot bind to actin to a nonrefractory state which can bind to actin. This model leads to several predictions which were studied in the present work. First, the fraction of heavy meromyosin or subfragment 1 which remains unbound to actin when the ATPase equals V_{max} should have the same properties as the original protein. In the present study it was determined that the unbound protein has normal ATPase activity which suggests that it is unbound to actin for a kinetic reason rather than because it is a permanently altered form of the myosin. Second, if the heavy meromyosin heads act independently half as much subfragment 1 as heavy meromyosin should bind to actin. Experiments in the ultracentrifuge demonstrate that about half as much subfragment 1 as heavy meromyosin sediments with the actin at V_{max}. Third, the ATP turnover rate per actin monomer at infinite heavy meromyosin concentration should be much higher than the ATP turnover rate per heavy meromyosin head at infinite actin concentration. This was found to be the case for SH_1-blocked heavy meromyosin since, even at very high concentrations of SH_1-blocked heavy meromyosin, in the presence of a fixed actin concentration, the actin-activated ATPase rate remained proportional to the SH_1-blocked heavy meromyosin concentration. All of these results tend to confirm the refractory state model for both SH_1-blocked heavy meromyosin and unmodified heavy meromyosin and subfragment 1. However, the nature of the small amount of heavy meromyosin which does bind to actin in the presence of ATP at high actin concentration remains unclear.

In muscle cells the key event in contraction is the interaction of myosin with actin and ATP at low ionic strength. In vitro kinetic studies of this interaction are difficult to interpret quantitatively because myosin aggregates at low ionic strength. However, heavy meromyosin and subfragment 1, which retain both the ATPase activity and actin-binding sites of myosin, are both soluble at low ionic strength. Their interaction with actin and ATP is, therefore, much more amenable to quanti-

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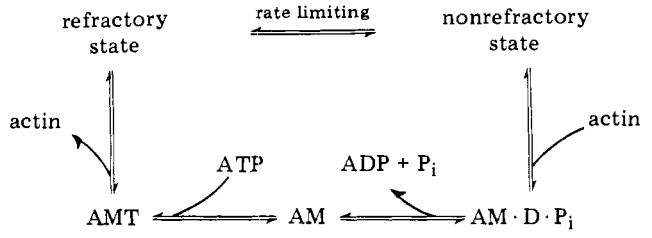
tative analysis. Such analysis has shown that, over a considerable range of ionic strength and temperature, there is a hyperbolic relationship between F-actin concentration and actin activation of the HMM[1] or S-1 ATPase (Eisenberg and Moos, 1970). However, analytical ultracentrifuge, viscosity, turbidity, quasi-electric-light scattering and kinetic studies have indicated that, even when the ATPase is close to V_{max}, the HMM and S-1 are still largely dissociated from the actin (Fraser et al., 1975; Eisenberg and Kielley, 1972).

When myosin is modified at the SH_1 group with *N*-ethylmaleimide only 3-fold actin activation is observed compared with 200-fold for normal HMM (Silverman et al., 1972). Nevertheless, it was found that there was still a hyperbolic relation between ATPase activity and actin concentration. Furthermore, ultracentrifuge, viscosity, and turbidity experiments indicated that, like normal HMM, the SH_1-blocked HMM was largely dissociated from the actin when the actin-activated ATPase was close to V_{max} (Mulhern et al., 1975).

To explain these findings, it was postulated that both normal and SH_1-blocked HMM undergo a rate-limiting transition from a refractory state which cannot bind to actin to a nonrefractory state which can bind to actin during the cycle of interaction with ATP and actin. Since the overall rate around the cycle is much slower for SH_1-blocked HMM than for normal HMM, it was suggested that the rate-limiting transition from the refractory to the nonrefractory state is much slower for SH_1-blocked HMM than for normal HMM. In schematic form the essentials of this model are as shown in Scheme I, where A = actin, M = myosin, T = ATP, and D = ADP.

Scheme I



This model leads to several predictions. First, the fraction of HMM or S-1 which remains bound to actin when the ATPase equals V_{max} should have the same properties as the original protein. It should not be a denatured or an altered form of the HMM or S-1. This is particularly important to demonstrate with SH_1-blocked HMM where it is quite possible that more than one species of modified HMM is present. Second, if the HMM heads act independently, at high actin concentration half as much S-1 as HMM should sediment with the actin; the binding of a single HMM head will cause both HMM heads to sediment in an ultracentrifuge experiment whereas the binding of a single S-1 head will not have this cooperative effect. Third, the ATP turnover rate per actin monomer at infinite HMM concentration should be much higher than the ATP turnover rate per HMM head at infinite actin concentration. This should occur because at infinite actin concentration, the rate of ATP hydrolysis per mole of HMM should be limited by the rate of the transition from the refractory to the nonrefractory state, whereas at infinite HMM concentration the rate of ATP hydrolysis per mole of actin will not be limited by the rate of this transition because there will be an infinite amount of HMM present in both the refractory and nonrefractory states. Qualitatively, this result was observed with normal HMM and S-1, but the extrapolated rate at infinite HMM concentration depended on the actin concentration, an unexpected result for which no explanation was obtained (Eisenberg and Kielley, 1972).

In the present study we tested these three predictions using both normal and SH_1-blocked HMM and S-1. Our results confirm that, for both the unmodified and modified myosin, when the ATPase is close to V_{max} most of the myosin heads remain unbound to actin, not because they are a denatured or altered form of the protein but because they are in the refractory state. Furthermore, ultracentrifuge studies with SH_1-blocked HMM show that the binding of the remaining myosin heads occurs only at very low ionic strength and does not correlate with the actin-activated ATPase activity. At the present time, we still do not have a definitive explanation for this binding.

Materials and Methods

Rabbit skeletal myosin was prepared by the method of Kielley and Harrington (1960), and HMM was prepared by the method of Eisenberg and Moos (1968). S-1 was prepared from the myosin using soluble papain as described by Lowey et al. (1969). Actin was prepared by a modified method of Spudich and Watt (1971) (Fraser et al., 1975). SH_1-blocked myosin was prepared by a modified method of Sekine and Kielley (1964) (Mulhern et al., 1975). Protein concentrations were determined by ultraviolet (UV) absorption using the extinction coefficients previously described (Mulhern et al., 1975). The extinction coefficient used for S-1 was $A_{280}^{\%} = 7.7$ cm^2/g (Young et al., 1965). Steady-state ATPase rates were measured with a Radiometer pH stat as previously described (Eisenberg and Moos, 1967).

Ultracentrifuge experiments were performed in a Model E analytical ultracentrifuge equipped with a photoelectric scanner as described by Eisenberg et al. (1972). For the separation cell experiments with unmodified HMM and S-1, a two-cell rotor was used with a fixed partition separation cell from Beckman on each side, one filled with solvent and one with the sample. A 12-mm Kel-F partition was positioned about one-third of the way up from the bottom of the sample cell. One hole in the partition was enlarged slightly so that the Teflon needle used to introduce the sample could fit through it. No filter paper was used on the perforated partition because we found that the presence of filter paper did not increase the effectiveness of the separation. Three separate ultracentrifuge runs were done for each experiment: one with actin alone, one with HMM or S-1 alone, and one with both actin and HMM or S-1. The rotor and cells were precooled to 0.5 °C before each run and were then centrifuged at 30 000 rpm, until the actin had sedimented below the separation barrier. The three runs were done for exactly the same length of time. In general, it took about 20 min from the time the centrifugation was started for the actin to sediment completely below the separation barrier. After centrifugation, approximately 0.15 ml of the sample remaining in the top of the separation cell was drawn off by means of a syringe with a Teflon needle. For the cell containing actin alone, the protein concentration of the sample was determined in duplicate by the method of Lowry et al. (1951). This protein concentration was subtracted from the protein concentration of the sample from the cell containing the reaction mixture, i.e., HMM or S-1 plus actin. However, because almost all of the actin sedimented in this experiment, this correction was never more than 20%. The other two sep-

[1] Abbreviations used: HMM, heavy meromyosin; S-1, subfragment 1; UV, ultraviolet; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

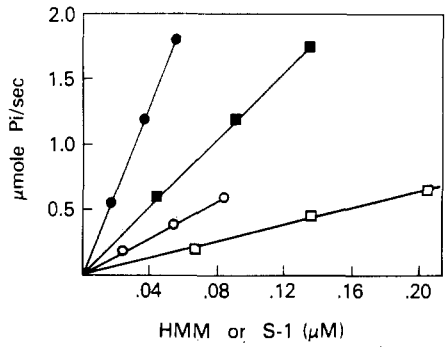


FIGURE 1: Standard curve of ATPase activity of HMM and S-1 under conditions of the separation cell experiment. HMM or S-1 solutions were made up in 3 mM ATP, 3 mM $MgCl_2$, 3 mM imidazole (pH 7.0), 2 mM P_i, i.e., the conditions of the separation cell sample. They were then diluted fivefold with 2 mM imidazole (pH 7.0). Protein and ATPase activity were determined as described under Materials and Methods for assay of the separation cell sample. Conditions for actin-activated ATPase assay: 0.1 ml HMM or S-1 solution prepared as above, 2 mM $AT^{32}P$, 2 mM $MgCl_2$, 10 mM imidazole (pH 7.0), 22 μM actin, 30 °C. The assays with S-1 were made 20 mM in KCl. Conditions for EDTA ATPase assay: 0.15 ml HMM or S-1 solution prepared as above, 2 mM $AT^{32}P$, 0.5 M KCl, 20 mM Tris (pH 7.5), 5 mM EDTA 30 °C. Note that the solution for the EDTA assay actually contained an additional 0.18 mM $MgCl_2$ because the original separation cell solution contained $MgCl_2$. (□, ■) S-1; (○, ●) HMM; (open symbols) EDTA ATPase; (filled symbols) actin-activated ATPase.

aration cell samples were diluted fivefold with 2 mM imidazole (pH 7) so that larger volumes could be used for easier handling and each was then divided into six parts. Protein concentrations were determined in duplicate on two parts and both EDTA and actin-activated ATPase assays were performed in duplicate on the remaining parts under the conditions described in the legend to Figure 1. ATPase assays were determined by the amount of $AT^{32}P$ hydrolyzed in 15 min in a total volume of 0.5 ml using a modified method of Martin and Doty (1949) to isolate $^{32}P_i$. The ATPase assay was terminated by the addition to the 0.5-ml sample of 0.25 ml of 2:5 mixture of 5 M H_2SO_4–6% silicotungstic acid followed by the addition of 1 ml of 1:1 2-butanol–benzene with stirring on a vortex mixer for about 10 s. Next 0.25 ml of 5% ammonium molybdate in water was added and the sample mixed again for 20 s. The phases were allowed to separate before 0.25 ml of the 2-butanol–benzene layer was removed, dissolved in 15 ml of Aquasol, and then counted in a Beckman LS-250 liquid scintillation system.

Standard plots were done for both the EDTA and actin-activated ATPase activity in exactly the same way as the assays on the separation cell samples themselves. In both cases the ATPase activity was determined over a protein concentration range that bracketed the concentration expected in the actual separation cell experiment (see Figure 1).

The separation cell experiment was performed with SH_1-blocked HMM in the same manner as for unmodified HMM and S-1 except that T = 15 °C, protein determinations were not made, and ATPase assays were performed in duplicate both in the absence and presence of actin under the conditions described in the legend to Table II.

N-Ethylmaleimide was purchased from Schwarz/Mann and resublimed before use. $AT^{32}P$ was obtained from ICN and diluted to 5.0 ml with 50 mM ATP (pH 7.0) and stored frozen.

Results

Separation Cell Experiment. In order to test if the presence of unbound HMM or S-1 at very high actin concentration (V_{max}) is due to its existence in the refractory state, the enzyme remaining unbound to actin at V_{max} was characterized and compared with the original enzyme, both in the presence and absence of salt. To demonstrate that this unbound enzyme is the same as the original enzyme and is unbound to actin only because it is in the refractory state, the ideal experiment would be to reisolate the unbound enzyme and show that, in a second exposure to actin, 40% of it sedimented with the actin as occurred in the first exposure. However, we were unable to recover enough enzyme to perform this experiment. Therefore, we took the next best approach and measured the ATPase activity of the unbound enzyme. In this way, we could determine if the enzyme were denatured or if the original preparation consisted of two separate species only one of which could bind to actin and show actin activation. This latter point is especially important for SH_1-blocked HMM where there may be more than one species of modified HMM present.

TABLE I: Characterization of HMM and S-1 Isolated from the Separation Cell.[a]

	Separation Cell Contents: % ATPase Activity			
	HMM	HMM + Actin	S-1	S-1 + Actin
Expt 1: EDTA ATPase	99	104	112	114
Actin ATPase	92	89	98	122
Expt 2: EDTA ATPase	107	94	118	104
Actin ATPase	103	92	108	99

[a] The analytical ultracentrifuge was run at 30 000 rpm at 0.5 °C. The separation cell contained 3 mM $MgCl_2$, 3 mM imidazole (pH 7.0), 2 mM P_i, 45 μM actin, and 5.7 μM HMM heads. After centrifugation the sample remaining in the top of the separation cell was assayed for protein and ATPase activity as described under Materials and Methods and in the legend to Figure 1. The calculated specific activity was then compared with Figure 1 where the slope of the standard curve was defined as 100% ATPase activity. A separate standard curve was run for each experiment.

To isolate the unbound enzyme we used the analytical ultracentrifuge equipped with a separation cell from which we could remove the protein remaining in the supernatant after the actin sedimented into the lower half of the cell. With unmodified HMM and S-1 the experiment was performed under essentially the same conditions as previously used in the ultracentrifuge experiments; i.e., the actin-activated ATPase was about 80% of V_{max} (Eisenberg and Kielley, 1972). The only difference in conditions in the separation cell experiment was that one-third as much HMM and S-1 were used to allow time for the actin to sediment below the separation cell barrier before all of the ATP was hydrolyzed. The protein isolated from the separation cell was divided into six fractions: two were used to measure the protein concentration, two were used to determine the EDTA ATPase, and two were used to determine the actin-activated Mg–ATPase. Since only a small amount of protein could be obtained from the separation cell during each run, the protein concentration was quite low in the assay mixtures. There have been reports that at low myosin concentration the myosin light chains dissociate from the heavy chains and the protein denatures, (Siemankowski and Dreizen, 1974) so it was important to show that the ATPase was a linear function of the concentration of HMM or S-1 in the assay mixture at the low protein concentrations used. The ATPase assays shown in Figure 1 were performed in exactly the same

TABLE II: Characterization of SH_1-Blocked HMM Isolated from the Separation Cell.[a]

Separation Cell Contents			ATPase Activity of Separation Cell Sample (μmol of ATP Hydrolyzed/30 min)		
Added NEM-HMM (μM)	Added Actin (μM)	KCl (mM)	No Actin in Sample	Actin in Sample	Actin Activation (-fold)
5.7	0	0	0.12	0.30	2.8
5.7	45	0	0.056	0.16	2.9
5.7	0	50	0.144	0.35	2.8
5.7	45	50	0.14	0.34	2.8

[a] The analytical ultracentrifuge was run at 30 000 rpm at 15 °C. The separation cell sample contained 3 mM ATP, 3 mM $MgCl_2$, 3 mM imidazole (pH 7.0), 2 mM P_i both in the absence and presence of 50 mM KCl and 45 μM actin. After centrifugation the sample remaining in the top of the separation cell was diluted fivefold with 2 mM imidazole and assayed for ATPase activity as described under Materials and Methods. Conditions for ATPase assay: 0.15 ml of SH_1-blocked HMM as prepared above, 2 mM $AT^{32}P$, 4 mM $MgCl_2$, 10 mM imidazole (pH 7.0), in the absence or presence of 45 μM actin, T = 25 °C.

way as the assays on the actual separation cell samples and, as can be seen, the EDTA and actin-activated ATPase activity of both the HMM and S-1 were linear over the range of protein concentration used. Such standard curves were determined for each separation cell experiment performed and their slopes were defined as 100% ATPase activity.

Table I shows, for two separate experiments, the ATPase activity of the HMM and S-1 remaining in the separation cell supernatant. In each experiment, one separation cell sample was run with actin and one without actin as a control. As can be seen for both the EDTA and actin-activated ATPase, the enzyme in the supernatant seemed to have the same activity as the original HMM or S-1 within a range of error of about 20%. This was true both for the control samples without actin present and for the experimental samples with actin present. It is, therefore, clear that the occurrence of HMM or S-1 bound to actin at V_{max} is not due to denaturation or to the presence of two species of HMM or S-1, only one of which binds to actin and has actin-activated ATPase activity. Rather, it must be due to HMM or S-1 heads passing through the refractory state during their cyclic interaction with ATP and actin.

Similar experiments were next performed with SH_1-blocked HMM. Since the ATPase rate of the SH_1-blocked HMM in the absence of actin is only one-third the rate in the presence of actin, we could compare the ATPase rates of SH_1-blocked HMM in the presence and absence of actin, a comparison which was not possible for unmodified HMM because its ATPase rate in the absence of actin was too low to accurately measure. Therefore, it was not necessary to determine the specific activity of the SH_1-blocked HMM, i.e., to measure its protein concentration. Rather, we only had to determine if the SH_1-blocked HMM in the separation cell supernatant still showed a threefold increase in ATPase activity when actin was added to the assay medium. The separation cell experiments were performed both in the absence of KCl, where the ATPase was 95% of V_{max} and about 60% of the HMM remained in the supernatant, and in the presence of 50 mM KCl, where the ATPase was 85% of V_{max} but almost all of the HMM appeared to remain in the supernatant. As can be seen in Table II, in both cases the SH_1-blocked HMM isolated in the separation cell supernatant showed about a threefold increase in activity in the presence of actin. Although exact specific activities were not determined, the rate of ATP hydrolysis was consistent with the amount of protein which was estimated to be present in the separation cell supernatant. These experiments show that the SH_1-blocked HMM which remains unbound to actin in the ultracentrifuge has the same properties as the original SH_1-blocked HMM. It is not denatured. Therefore, the actin-activated ATPase is not due to the occurrence of a small amount of a separate species of HMM which sediments with the actin leaving the remaining HMM in the supernatant.

Comparison of HMM and S-1 Binding to Actin. If the two HMM heads act independently, it might be expected that, in the presence of excess actin under conditions where the same number of HMM as S-1 heads bind to actin, roughly twice as many moles of HMM as S-1 would sediment with the actin. This would occur because the binding of one of the HMM heads would carry the second head down with it, a cooperative effect which would not occur with the single-headed S-1. On this basis the binding to actin of 20% of the heads present might be expected to cause 40% of the HMM but only 20% of the S-1 to sediment.

Before determining whether this was, in fact, the case, we made certain that the sedimentation process, itself, did not affect the binding of the HMM to the actin by testing the effect of g force on this binding. These studies were carried out under the same conditions as our previous analytical ultracentrifuge experiments in the absence of KCl where the ATPase equaled 87% of V_{max} (Eisenberg and Kielley, 1972). As previously, the protein in the supernatant was estimated from the analytical ultracentrifuge scanner trace. The results indicate that over a fourfold range of g force (26 000 to 58 000 rpm) the amount of HMM sedimenting remained approximately 34%. Therefore, there seems to be no effect of sedimentation rate on the amount of protein bound to actin.

Next we compared, at varying actin concentrations, the amount of unmodified HMM and S-1 bound to actin in the presence of ATP. As shown in Figure 2 the amount of S-1 sedimenting was always less than the amount of HMM sedimenting and, as the ATPase approached V_{max}, about 20% of the S-1 bound to the actin compared with about 40% of the HMM. A similar result was obtained with the SH_1-blocked HMM and S-1. As shown in Table III, in the absence of KCl 25% of the SH_1-blocked S-1 bound to the actin compared with about 40% of the SH_1-blocked HMM. In 50 mM KCl, as previously reported for SH_1-blocked HMM, neither the SH_1-blocked S-1 nor the SH_1-blocked HMM showed measurable binding to the actin when the ATPase was near V_{max}. Due to the relatively small amount of binding which occurred in these experiments with both unmodified and SH_1-blocked S-1, these results are not exact. Nevertheless, in many dupli-

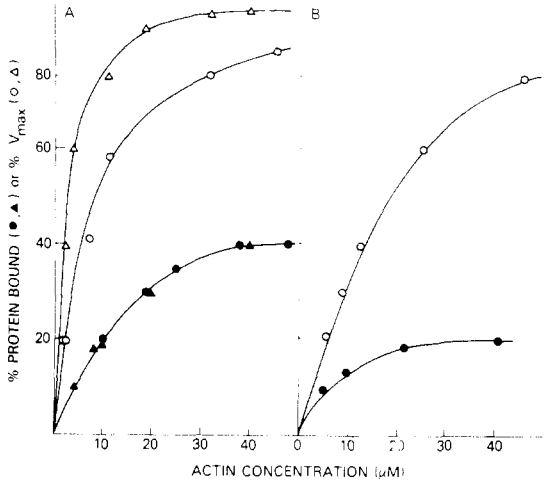


FIGURE 2: ATPase activity and binding of HMM and S-1 to actin at varying actin concentrations. (A) The binding of HMM (●) and SH_1-blocked HMM (▲) to actin at varying actin concentrations. The analytical ultracentrifuge was run at 30 000 rpm as described by Eisenberg et al. (1972). Conditions: 3 mM ATP, 3 mM $MgCl_2$, 2 mM P_i, 3 mM imidazole (pH 7.0), 5.8 μM HMM, or SH_1-blocked HMM, T = 0.5 °C for HMM and T = 15 °C for SH_1-blocked HMM. The ATPase activity of HMM (○) or SH_1-blocked HMM (△) as determined on the pH stat under the same conditions as the binding experiment. (B) The binding (●) and ATPase activity (○) of unmodified S-1 under the same conditions as A, except 11.6 μM unmodified S-1 was added.

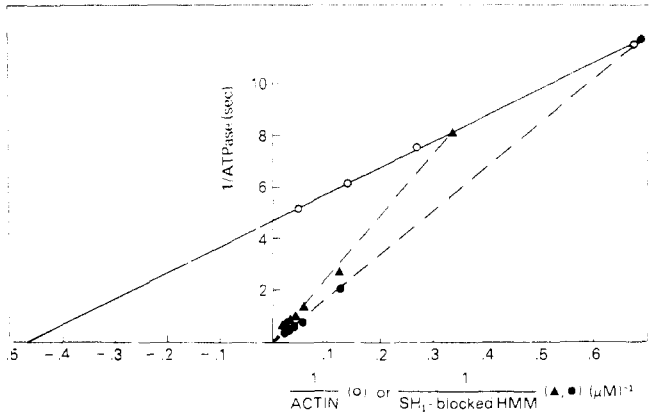


FIGURE 3: Double-reciprocal plots of acto-SH_1-blocked HMM ATPase rate vs. added actin concentration or SH_1-blocked HMM concentration in the presence of 25 mM KCl. Conditions: 2 mm ATP, 4 mM $MgCl_2$, 3 mM imidazole (pH 7.0), 25 mM KCl, T = 15 °C. The ATPase rate is corrected for the SH_1-blocked HMM ATPase rate alone which was 0.05 s^{-1}. (○) With 5.8 μM SH_1-blocked HMM, actin concentration varied; (●) 1.5 μM actin, SH_1-blocked HMM concentration varied; (▲) 3.0 μM actin, SH_1-blocked HMM concentration varied.

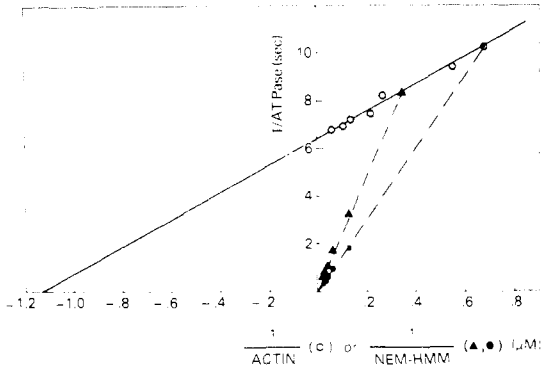


FIGURE 4: Double-reciprocal plots of acto-SH_1-blocked HMM ATPase rate vs. added actin concentration or SH_1-blocked HMM concentration in the absence of salt. Conditions as in Figure 3 except no KCl was added. The ATPase rate is corrected for the SH_1-blocked HMM ATPase rate alone, which was 0.02 s^{-1}. (○) With 5.8 μM SH_1-blocked HMM, actin concentration varied; (●) 1.5 μM actin, SH_1-blocked HMM concentration varied; (▲) 3.0 μM actin, SH_1-blocked HMM concentration varied.

TABLE III: Amount of SH_1-Blocked HMM and S-1 Bound to Actin.[a]

Protein	KCl (mM)	% V_{max}	% Bound
SH_1-blocked HMM	0	95	40
SH_1-blocked S-1	0	90	25
SH_1-blocked HMM	50	85	0
SH_1-blocked S-1	50	85	0

[a] The % V_{max} of S-1 was calculated from double-reciprocal plots as described previously for SH_1-blocked HMM (Mulhern et al., 1975). The binding experiments were performed in the analytical ultracentrifuge as described previously (Eisenberg et al., 1972). 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 or S-1, 40 μM actin, T = 15 °C.

cate experiments, it was always clear that considerably less S-1 than HMM sedimented with the actin.

Kinetic Studies. At both 25 mM KCl (Mulhern et al., 1975) and 50 mM KCl (Table III), almost all of the SH_1-blocked HMM or S-1 is dissociated from the actin when the ATPase is close to V_{max}. This implies that, under these conditions, the only slow step in the cycle of ATP hydrolysis is the transition from the refractory to the nonrefractory state while all of the other steps are considerably faster. This in turn means that almost all of the SH_1-blocked HMM will accumulate directly in front of this rate-limiting step. The HMM will all be in the refractory state dissociated from the actin, which, in turn, implies that all of the actin will be free of HMM.

If, at a fixed actin concentration, we increase the HMM concentration, and almost all of the actin remains free, the actin-activated ATPase rate per actin monomer should be proportional to the SH_1-blocked HMM concentration. On the other hand, if the actin becomes saturated with SH_1-blocked HMM, the ATPase rate per actin monomer will level off as the SH_1-blocked HMM concentration is increased. Thus, by increasing the SH_1-blocked HMM concentration at fixed actin concentration, we can determine whether, as suggested by ultracentrifuge experiments, the SH_1-blocked HMM and actin are almost completely dissociated at 25 mM KCl.

The open circles in Figure 3 show that, as we previously reported, at a fixed concentration of SH_1-blocked HMM with 25 mM KCl present, a double-reciprocal plot of the actin-activated ATPase activity vs. actin concentration is linear (Mulhern et al., 1975). The dashed lines in Figure 1 show what would theoretically occur if, at two fixed actin concentrations, the ATPase rate per actin monomer were proportional to the added SH_1-blocked HMM concentration. The closed symbols show the actual experimental data. In all of these experiments, the ATPase rate of the SH_1-blocked HMM alone is subtracted from the actin-activated ATPase rate which is about threefold higher. As can be seen, even at quite high SH_1-blocked HMM concentrations, the ATPase rate per actin monomer is proportional to the HMM concentration. This result is observed with both actin concentrations studied. These results are in agreement with ultracentrifuge experiments showing that, in 25 mM KCl, the actin and SH_1-blocked HMM are almost

completely dissociated. Figure 4 shows a similar experiment in the absence of KCl. Here one might expect a somewhat different result since the ultracentrifuge experiment showed that at V_{max} more than 40% of the HMM bound to actin in the absence of salt. In fact, however, the result is essentially the same as in 25 mM KCl; i.e., the ATPase rate per actin monomer is proportional to the SH_1-blocked HMM concentration for both actin concentrations studied.

Discussion

Three basic questions arise in our studies on the binding of unmodified and SH_1-blocked HMM to actin in the presence of ATP. First, is the presence of unbound protein due to the existence of a refractory state through which all of myosin heads pass as they interact with actin and ATP, or is it due to a denatured or altered form of the myosin? Second, exactly how much HMM and S-1 are bound to actin at V_{max}? Third, what is the nature of this binding?

The separation cell experiments presented in this paper show that, for both the unmodified and the SH_1-blocked HMM and S-1, the protein which remains unbound to actin when the ATPase equals V_{max} has normal ATPase activity. This, in turn, strongly suggests that it is unbound to actin for a kinetic reason rather than because it is a denatured or permanently altered form of the myosin. Therefore, the separation cell experiments support the idea that most of the HMM and S-1 heads are in the refractory state when the actin-activated ATPase is close to V_{max}. Recent stopped-flow experiments from our laboratory showing a single cycle of ATP hydrolysis of acto-S-1 also support this conclusion (Chock et al., 1976).

As for quantifying the amount of protein bound to the actin at high actin concentration, this remains a difficult problem. With both unmodified and SH_1-blocked HMM in the absence of KCl, the analytical ultracentrifuge experiments suggest that about 40–50% of the HMM sediments with the actin. This binding is probably not an artifact of the ultracentrifuge measurement since it is always accompanied by an increase in the viscosity of the acto-HMM or acto-S-1 solution. Unfortunately, viscosity is a nonlinear measure of binding and thus cannot be used to quantify the amount of HMM bound.

A simple explanation for this binding is that, during each cycle of ATP hydrolysis, a complex forms between actin and one of the HMM heads which has randomly transformed to the nonrefractory state. At high actin concentration, the steady-state concentration of this complex could cause the observed binding. In agreement with this hypothesis, at high actin concentration, roughly half as much S-1 as HMM binds to the actin. This is consistent with (but does not prove) the concept that the HMM heads act independently since the binding of one of the HMM heads which had transformed to the nonrefractory state would carry down the second head even if it remained in the refractory state.

Unfortunately, this simple explanation for the binding is not supported by some of our other data. One difficulty is that the observed binding does not always correlate with the actin-activated ATPase activity. The data in Figure 2 show that, as the actin concentration is increased, the binding of unmodified HMM and S-1 to actin and the actin-activated ATPase rate increase in a parallel manner. However, this correlation between binding and ATPase is probably fortuitous because, while the binding of SH_1-blocked HMM to actin in the absence of KCl shows this same dependence on actin concentration (closed triangles, Figure 2), the actin-activated ATPase of the SH_1-blocked HMM (open triangles, Figure 2) shows a quite different dependence on actin concentration, leveling off at a much lower actin concentration. Furthermore, at 25 or 50 mM KCl both ultracentrifuge and viscosity studies suggest that the actin and SH_1-blocked HMM are almost completely dissociated even when the actin-activated ATPase is 90% of V_{max} (Mulhern et al., 1975). All of these results suggest that the amount of HMM or S-1 bound to actin in the absence of salt does not necessarily correlate with the actin-activated ATPase activity as would be expected from the simple hypothesis given above.

Another difficulty with this hypothesis is that viscosity, turbidity, and kinetic measurements all suggest that much less HMM and S-1 are bound to actin than in the ultracentrifuge experiments. Previous viscosity and turbidity studies suggested that about 10% of the S-1 and even less HMM was bound to actin at V_{max} (Fraser et al., 1975), although, as we mentioned above, the viscosity studies do qualitatively agree with the ultracentrifuge results. One possible explanation for these data is that physical studies of this type are nonlinear measures of binding so that, particularly with HMM, they underestimate the amount of binding. However, the results of the kinetic studies presented in this paper cannot be explained in this way. We find that the actin-activated ATPase of the SH_1-blocked HMM is proportional to the concentration of the SH_1-blocked HMM even at very high HMM concentration both in the presence and absence of salt. This result suggests that the actin remains free to react with the SH_1-blocked HMM even when this HMM is present in great excess over the actin. In other words very little complex is formed between the actin and SH_1-blocked HMM even at very high SH_1-blocked HMM concentration. On the other hand, as we discussed above, at high actin concentration in the absence of salt, ultracentrifuge experiments suggest that 40–50% of the SH_1-blocked HMM binds to actin. A similar result occurred with unmodified HMM where kinetic studies indicated that less than 20% of the HMM bound to actin at very high HMM concentration whereas ultracentrifuge experiments suggested that 40–50% of the HMM was bound to actin at high actin concentration (Eisenberg and Kielley, 1972). The results with unmodified HMM were complicated by a dependence of the extrapolated ATPase rate at infinite HMM concentration on actin concentration. As can be seen in Figures 3 and 4, this unexplained dependence on actin concentration does not seem to occur with SH_1-blocked HMM. Nevertheless, at the present time we cannot easily reconcile our kinetic and ultracentrifuge observations with SH_1-blocked HMM. Both observations support our overall conclusion that the HMM heads pass through a refractory state during each cycle of ATP hydrolysis. But further work will be necessary to understand the exact nature and extent of the binding to actin which does occur in the absence of KCl with both unmodified and SH_1-blocked HMM.

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Effects of the Aliphatic Carboxylate Series of Salts on the Conformation of Proteins†

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ABSTRACT: The effects of the aliphatic acid series of salts, formate, acetate, propionate, butyrate, valarate, and caproate, on the conformation of sperm whale myoglobin, human hemoglobin A, and horse heart cytochrome *c* were investigated by spectral measurements in the Soret region, optical rotation, and intrinsic viscosity measurements. The effectiveness of the aliphatic acid salts as unfolding reagents for proteins is found to increase with increasing hydrocarbon content of the alkyl chains of the salts, which is analogous in behavior to effects of the urea, amide, and alcohol series of protein denaturants. The denaturation midpoints, S_m, as a function of the unfolding reagent were analyzed using the equations of Peller (Peller, L. (1959), *J. Phys. Chem. 63,* 1199) and Flory (Flory, P. J. (1957), *J. Cell. Comp. Physiol. 49,* 175) with binding constants based in part on the Scheraga–Nemethy theory of hydrophobic bonding or evaluated from free-energy transfer data of nonpolar amino acid side chains from aqueous to nonaqueous solvents. The summation of the polar K_P and hydrophobic $K_{H\Phi}$ contributions of solvent to protein amino acid side chain interactions were found to give best account of the protein denaturation data. Intrinsic viscosity and optical rotation data obtained on hemoglobin and myoglobin at high salt concentrations, above the unfolding transition regions, indicate that the product of denaturation by the aliphatic acid salts is less unfolded than in 6 M guanidine hydrochloride solutions. Residual elements of the helical regions of the proteins seem to either escape unfolding or are reformed at high concentrations of the denaturing salts.

A variety of organic solutes and electrolytes are known to alter the native conformation of proteins and other biopolymers by producing changes in the structure in the supporting natural solvent, water, or by preferential interaction with the biopolymer constituents (Kauzmann, 1959; Tanford, 1970; Noelken, 1970; Cann, 1971). Studies from our laboratory in the past several years have dealt with both the effects of the hydrophobic series of organic solutes, i.e., the ureas, the amides, and the alcohols, on the native conformation of proteins (Herskovits and Jaillet, 1969; Herskovits et al., 1970a–c; Elbaum et al., 1974; Herskovits and Solli, 1975; Herskovits and Harrington, 1975) and their effects on the state of association of subunit proteins such as the hemoglobins (Elbaum and Herskovits, 1974; Bhat and Herskovits, 1975; Harrington and Herskovits, 1975). The theories of Peller (1959) and Flory (1957) developed to account for the effects of salts and neutral additives on the temperature transitions of biopolymers were used in our denaturation studies in conjunction with binding constants based on free-energy transfer data and the Scheraga–Nemethy theory of hydrophobic bonding (Schrier et al., 1965). Group additivity of the polar and hydrophobic constituents of the denaturant, suggested by the model studies of Schrier and Schrier (1967) and Nandi and Robinson (1972a,b), was assumed for the evaluation of the binding constants in these studies (Herskovits et al., 1970c; Elbaum et al., 1974) as well as in our studies on DNA denaturation (Herskovits and Harrington, 1972; Herskovits and Bowen, 1974) and hemoglobin dissociation (Elbaum and Herskovits, 1974; Bhat and Herskovits, 1975; Herskovits and Harrington, 1975).

As a means of bridging the effects of electrolytes and hydrophobic solutes on the conformation and subunit organization of biopolymers and as a further test of group additivity of

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