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The Interaction of Heavy Meromyosin and Subfragment 1 with Actin. Physical Measurements in the Presence and Absence of Adenosine Triphosphate†

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ABSTRACT: Viscosity, turbidity, and laser-light fluctuation autocorrelations of acto-heavy meromyosin (HMM) and acto-subfragment 1 (S-1) solutions were measured under conditions where the actin-activated ATPase is close to its maximal value. The results were compared to similar data obtained in the absence of ATP where the actin and myosin fragments were completely complexed, and in the presence of ATP but at 0.1 *M* KCl where the actin and HMM or S-1 were almost completely dissociated. It was found that at maximal actin activation, the viscosity, turbidity, and autocorrelation data were all much closer to the values for the completely dissociated systems than to the values for the completely complexed systems. Assuming that viscosity, turbidity, and autocorrelation measurements approximate a linear measure of binding between actin and HMM or S-1, the results suggest that at maximal actin activation less than 10% of the HMM or S-1 are bound to the actin. Therefore as was suggested previously by ultracentrifuge and kinetic studies, it appears that under conditions of maximal actin activation, most of the HMM and S-1 occur in a refractory state unable to bind to actin.

A key question in the biochemistry of muscle contraction is the nature of the interaction between actin and myosin in the presence of ATP. In vivo X-ray diffraction studies suggest that in the absence of ATP, more than 90% of the myosin bridges are attached to the F-actin filaments (Huxley, 1968), while in the presence of ATP, in activated muscle, less than 50% of the bridges appear to be attached (Miller and Tregear, 1970; Haselgrove and Huxley, 1973). In vitro studies also demonstrate that a marked difference occurs in the binding of actin to myosin in the presence and absence of ATP. In the absence of ATP, the actin–HMM[1] binding is essentially stoichiometric with maximum binding occurring at a molar ratio of two F-actin monomers/HMM molecule (Eisenberg et al., 1972; Margossian and Lowey, 1973). On the other hand, Perry and his coworkers concluded more than 10 years ago from physical measurements that the actin and HMM are almost completely dissociated in the presence of ATP even at very low ionic strength (Leadbeater and Perry, 1963; Perry et al., 1966). Of course these observations could have been due to the occurrence of a weak binding constant between actin and HMM in the presence of ATP, in which case presumably, had the actin concentration been raised to a high enough level all of the HMM would have become complexed with actin. At low ionic strength, however, actin activates the HMM ATPase and, furthermore, this actin activation follows a simple hyperbolic plot so that at very low salt concentration, experiments can be performed at actin concentrations where the actin activated HMM ATPase is nearly at its maximum value (Rizzino et al., 1970; Eisenberg et al., 1972; Eisenberg and Kielley, 1972). At these actin concentrations weak binding between actin and HMM cannot be invoked as an explanation for the dissociation of the actin and HMM. Rather, at saturating actin concentration, the actin and

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[1] Abbreviations used are: HMM, heavy meromyosin; S-1, subfragment 1.

HMM should be completely associated even if the binding between the actin and HMM is relatively weak. Recently, however, Eisenberg et al. (1972; Eisenberg and Kielley, 1972) found that even under conditions where actin maximally activates the HMM or S-1 ATPase, the acto-HMM or acto-S-1 are still markedly dissociated as shown by ultracentrifuge and kinetic experiments. On this basis they proposed that in the presence of ATP, the myosin head can exist in two conformations, the chemical natures of which are as yet undetermined. One of these conformations they called the refractory state since it seemed to be unable to bind to actin at any concentration of actin obtainable in vitro. The other conformation they called the nonrefractory state since presumably it is able to bind to actin and thus accounted for the actin activation of the HMM ATPase.

In the present study we again studied the interaction of actin and HMM in the presence of ATP under conditions where actin nearly maximally activates the HMM ATPase. In this paper, however, we measured viscosity, turbidity, and intensity fluctuation autocorrelation to determine the amount of HMM or S-1 bound to actin. Our results suggest that as was indicated by the previous ultracentrifuge and kinetic studies, a large fraction of the HMM and S-1 remains unbound to actin under conditions where actin maximally activates the HMM or S-1 ATPases, i.e., a large fraction of the HMM or S-1 seems to be in the refractory state.

Methods

Protein Preparation. Rabbit proteins were used. Myosin was prepared by the method of Kielley and Harrington (1960). HMM was prepared from the myosin as described by Eisenberg and Moos (1967). The method of Lowey et al. (1969) was used to make S-1. Actin was prepared by a modification of the method of Spudich and Watt (1971; Eisenberg and Kielley, 1974). The resulting G-actin in 0.5 m*M* ATP, 0.1 m*M* Ca, 2 m*M* Tris (pH 8), and 0.1 m*M* 2-mercaptoethanol was polymerized by making it 0.1 *M* KCl, 3 m*M* Mg, and 5 m*M* P_i. It was then dialyzed for 2 days against 3 m*M* Mg, 5 m*M* P_i, and 0.5 m*M* ATP to remove the KCl, and then used without further removal of the ATP. This method was followed to avoid centrifugation followed by resuspension of the pellet by homogenization, a procedure which tended to give a relatively turbid F-actin preparation which was unsuitable for the quasielastic light scattering measurements.

Experimental Procedures. The viscosity, turbidity, and intensity fluctuation autocorrelation studies were all performed under the conditions described by Eisenberg et al. (1972), i.e., 0–1°, 3 m*M* imidazole and 2 m*M* P_i (pH 7.0), 3 m*M* $MgCl_2$, initial ATP concentration 3 m*M*, and total protein concentrations 1–2 mg/ml. To prevent actin–HMM interaction in control experiments 0.1 *M* KCl was added which greatly reduced the actin-activated ATPase. Acto-HMM and acto-S-1 gels in the absence of ATP were prepared by allowing all of the added ATP to be hydrolyzed to ADP and P_i prior to measurement. At very low HMM or S-1 concentrations this hydrolysis was performed at 30° and then the temperature was lowered to 0° for the measurements.

Viscometry. Viscosity was measured with Ostwald viscometers with 6-ml sample chambers and water outflow times of 15.6–20.6 sec at 0.5°. The samples were pipetted into viscometers in a 0.5° water bath and were then repeatedly drawn to the starting positions and allowed to flow out. The outflow times were converted to relative viscosities, η_{rel}, and the log η_{rel} was calculated. Comparisons of the viscosity of various mixtures with the viscosity of their individual components were then made using the theoretical relationship for noninteracting components A and B (Szent-Gyorgyi, 1951):

$$\log (\eta_{rel\ A+B}) = \log (\eta_{rel\ A}) + \log (\eta_{rel\ B}) \qquad (1)$$

Turbidity. Turbidities were measured by placing the samples in a cuvet thermostated at 0.5° in a Cary double beam spectrometer operating at 350 nm. Both the entrance and exit apertures were 0.15 mm.

Intensity Fluctuation Autocorrelation. The diffusive behaviors of the mixtures were estimated by intensity fluctuation autocorrelation (Cummins and Swinney, 1970). The experimental arrangement used was described by Carlson et al. (1972), with the exception that "prescaling" (Jakeman et al., 1972) was used instead of "single clipping". Protein solutions were placed at the scattering center in 1-cm cuvets which were thermostated to 0–1°. Measurements were made in 100-sec time samples at external scattering angles of 63.80°, 90°, and 116.2° using 457.9-nm radiation. Time autocorrelation functions of the scattered light were generated in a digital correlator (Fraser, 1971). The autocorrelation functions were compared by direct observation and by fitting average line widths, $\bar{\Gamma}$, by the method of Koppel (1972). Analysis was cut off at $0.22[g^{(2)}(0) - g^{(2)}(\infty)] + 1$. A line width obtained by the Koppel method provides the Z-average diffusion coefficient in polydisperse simple mixtures of Rayleigh scatterers.

The complex scattering and diffusive properties of F-actin precluded the usual way of determining diffusion constants by plotting line width vs. the square of the reciprocal of the scattering vector. This is caused by several nonidealities: apparent partial aggregation of F-actin (as shown by great turbidity and uneven pouring); the diffusive properties of approximately 1 μm long F-actin molecules at 1 mg/ml which can occupy only 0.03 μm^3 of solution per particle; and the uncertain Mie scattering of actin molecules of varying lengths and unknown contour shape. These problems were avoided in this study by performing experiments at fixed angles and finding trends at each scattering angle as a function of the molecular components of the system. The same trends appeared at each angle and on this basis conclusions were drawn. (The flexibility and diffusion of F-actin systems have been discussed by Fujime and Ishiwata (1971, Ishiwata and Fujime, 1972).) Our results are in disagreement, as the data below will imply. A note discussing these discrepancies is submitted elsewhere (Carlson and Fraser, 1974).

Brief Introduction to Intensity Fluctuation Autocorrelation. If coherent light is scattered from any group of objects, a pattern of scattered intensities about the scattering center is created. This is the diffraction pattern of the scatterers. It arises because there are different path lengths traversed from the light source to the various scattering elements and then to each point in the diffraction pattern. If the path lengths are such that the waves of light from the various scattering elements are in phase, there will be a bright spot. If the path lengths are such that the phases are exactly opposite, there will be darkness.

Molecules in a solution scatter light. When coherent laser light is scattered by a group of molecules, a diffraction pattern is generated. The molecules are in random positions, so their diffraction pattern is a random pattern of relatively bright and dim spots. Since the molecules are diffusing,

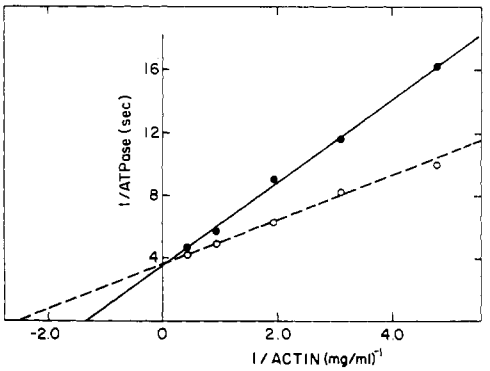

FIGURE 1: Dependence of HMM and S-1 ATPases on actin concentration. Conditions: 3 m*M* MgATP; 2 m*M* P_i; 3 m*M* imidazole (pH 7.0), temperature, 0.5°; (○) 2.1 mg/ml of HMM; 1/ATPase = [μmol of P_i/(μmol of HMM head sec)]$^{-1}$ assuming mol wt of HMM head = 175,000; (●) 1.5 mg/ml S-1; 1/ATPase = [μmol of P_i/(μmole S-1 sec)]$^{-1}$ assuming mol wt of S-1 = 120,000.

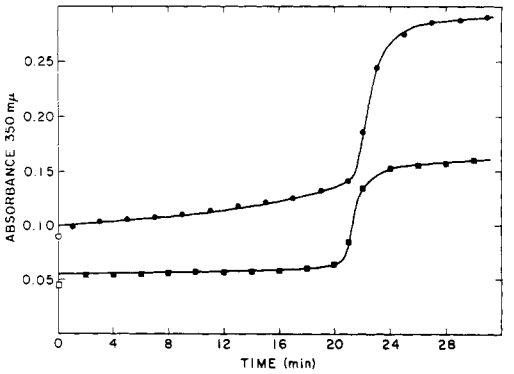

FIGURE 2: Turbidity of acto-HMM and acto-S-1. Conditions: 3 m*M* MgATP; 2 m*M* P_i; 3 m*M* imidazole (pH 7.0); 1 mg/ml of actin; temperature, 0.5°; (●) 2 mg/ml of HMM; (■) 1.35 mg/ml of S-1; (○) sum of turbidities of actin and HMM measured individually; (□) sum of turbidities of actin and S-1 measured individually.

their changing random positions lead to a changing random diffraction pattern. The light scattered to a fixed point in the diffraction pattern randomly varies in intensity as the phases of the scattered light from the moving scatterers become more and less reinforcing.

The temporal properties of the changing intensity at a spot in a diffraction pattern is the experimental observable of intensity fluctuation autocorrelation (or quasielastic light scattering). If the scattering elements never move, the light intensity at any point is constant; if the scattering elements move, the resulting phase changes cause a fluctuating intensity; if the particles move more rapidly, the fluctuations are more rapid. In the case of simple diffusion, the mean time required for the decay of a fluctuation is $(2D_Tq^2)^{-1}$, where D_T is the translational diffusion coefficient and q is $4\Pi n_0\lambda^{-1} \sin \psi/2$, where n_0 is the index of refraction of the solvent, λ is the wavelength of the light in vacuo, and ψ is the angle between the incident and scattered light. In general, the time required for fluctuations is smaller at larger scattering angles, ψ, because geometrical calculations show the light phase differences for fixed displacements to be greater at larger ψ.

We measured the time autocorrelation of the scattered light intensity. This is a measure of the magnitude and rapidity of the fluctuation of the scattered light. Mathematically, the intensity autocorrelation function is the average value of the intensity times the intensity at a time τ later. The average is performed over all possible starting times. So

$$\text{intensity autocorrelation} = \langle I(t)I(t+\tau)\rangle \qquad (2)$$

where $I(t)$ is the instantaneous intensity of light and the fences indicate time averaging. The autocorrelation function of the fluctuations contains the same information as the power spectrum that is measured in other investigations; the two are a Fourier transform pair.

The autocorrelation functions of necessity start at some maximum value and, in the case of chaotic random signals, they decay to an asymptote. That asymptote is $\langle I(t)\rangle^2$, is calculable, and is usually deleted from presented data by dividing the autocorrelation function by it and then subtracting 1. The resulting normalized autocorrelation function is then

$$g^{(2)}(\tau) - 1 = (\langle I(t) - I(t+\tau)\rangle/\langle I(t)\rangle^2) - 1 \qquad (3)$$

If an autocorrelation function has almost the same amplitude at the maximum as at the assymptote, it is because the scattering elements are not able to move distances that appreciably alter the phase of the scattered light, i.e., they are bound to remain within about 1 μm as occurs with acto-HMM gels.

If one intensity fluctuation autocorrelation function decays more rapidly to its asymptote than another one made at the same ψ, it must be because its moving scatterers are moving more rapidly. In simple cases of a single diffusing Rayleigh scattering species, the normalized autocorrelation function is an exponential curve of the form e^{-t}/τ_0 or $e^{-\Gamma t}$, where τ_0 is the time constant of the system and Γ, the bandwidth, is its reciprocal. More complex systems are often fitted with weighted average bandwidths, $\bar{\Gamma}$. Therefore mean bandwidth $\bar{\Gamma}$ is simply a measure of the rate of decay of the normalized autocorrelation function, i.e., the larger it is, the faster the scatterers are moving.

Results

Turbidity Studies. Perhaps the simplest measure of binding between HMM and actin is turbidity or absorbance at 350 mμ. In a recent study, Lymn and Taylor (1971) used turbidity as a measure of the amount of acto-HMM complex present, equating the rate of decrease in turbidity with the rate of decrease in the amount of complex. It is therefore of considerable interest to determine the turbidity of acto-HMM in the presence of ATP under conditions where actin maximally activates the HMM ATPase.

Figure 1 shows double reciprocal plots of HMM and S-1 ATPase vs. actin concentration at 0° with no added KCl, a condition where the actin activated ATPase reaches its maximum value at relatively low actin concentrations. Under these conditions at 1 mg/ml of actin concentration, the acto-HMM ATPase is 73% of V_{max} and the acto-S-1 ATPase is 60% of V_{max}. Parenthetically it is interesting to note that the turnover rate per mole of HMM head and that per mole of S-1 are nearly identical, in agreement with a similar study of Margossian and Lowey (1973).

Figure 2 shows the turbidity of acto-HMM and acto-S-1 under the above conditions where the actin-activated HMM and S-1 ATPase are approaching V_{max}. The open symbols on the ordinate show the sum of the turbidity of HMM or S-1 and that of actin, i.e., the theoretical turbidities which would be expected if no interaction were occurring between the actin and myosin subunits and dilute solution light scat-

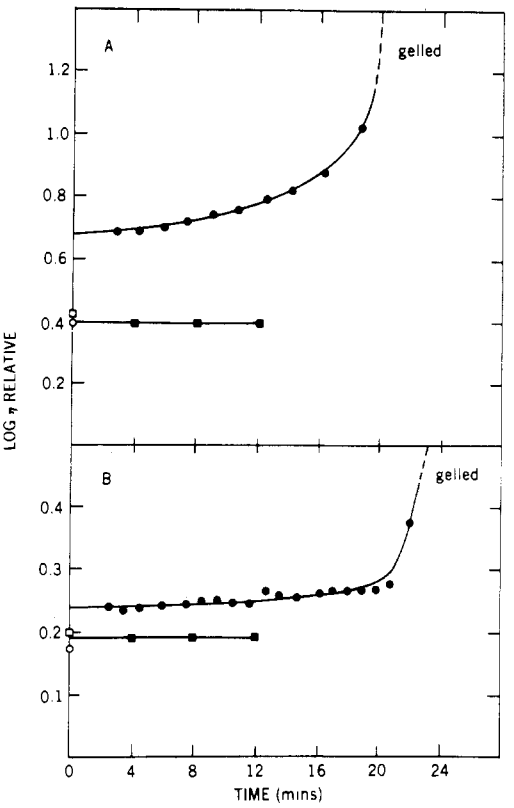

FIGURE 3: Viscosity of acto-HMM and acto-S-1. Conditions: 3 m*M* MgATP; 2 m*M* P_i; 3 m*M* imidazole (pH 7.0); temperature, 0.5°; (A) 2 mg/ml of actin, 2 mg/ml of HMM; (B) 1 mg/ml of actin; 1.35 mg/ml of S-1; (■) 0.1 *M* KCl; (●) no added KCl; (□) sum of the log of $\eta_{relative}$ of actin and HMM (A) and S-1 (B) measured individually at 0.1 *M* KCl; (○) sum of the log η_{rel} of actin and HMM (A) and S-1 (B) measured individually in the absence of KCl.

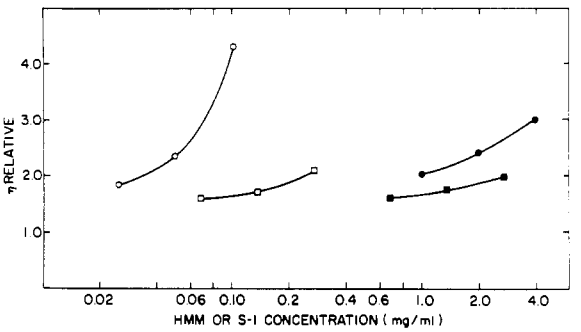

FIGURE 4: Comparison of the viscosities of acto-HMM and acto-S-1 with and without ATP. Conditions: (■,$\bullet_1$) 3 m*M* MgATP, 2 m*M* P_i; 3 m*M* imidazole (pH 7.0); 1 mg/ml of actin, temperature, 0.5°; (□,○) same conditions except ATP completely hydrolyzed to ADP and P_i; (●,○) HMM added; (■,□) S-1 added.

tering laws were applicable. The solid circles and squares, on the other hand, show the turbidity of acto-HMM and acto-S-1, respectively, under conditions where the ATPase is 60–75% of V_{max}. As can be seen in the presence of ATP at the beginning of the reaction, the turbidity is increased only very slightly over the theoretical value expected in the absence of interaction between actin and the myosin subunits. This contrasts markedly with the turbidity following hydrolysis of all of the ATP. At 25 min with the ATP completely hydrolyzed the turbidity shows a very marked increase above the value expected for no interaction. Presumably under this condition all of the HMM is bound to actin. Therefore if turbidity approximates a linear measure of the binding between the myosin subunits and actin, these data suggest that very little binding of HMM or S-1 to actin occurs, even when the actin activation of the ATPase is close to V_{max}.

Viscosity Studies. Viscosity is another physical measurement which can be used to detect an interaction between the myosin subunits and actin. The solid squares in Figure 3A show the viscosity at 0.1 *M* KCl where due to the relatively high ionic strength little actin activation of the HMM ATPase occurs and the actin and HMM are almost completely dissociated. The open square on the ordinate shows the viscosity expected under this condition in the absence of any interaction between the actin and HMM and as can be seen the measured values are essentially equal to the theoretical value at this ionic strength. On the other hand, at very low ionic strength where there is nearly maximum actin activation of the HMM ATPase a different result is obtained. The open circle on the ordinate in Figure 3A shows the theoretical viscosity expected in the absence of interaction between the actin and HMM whereas the solid circles show the experimentally measured viscosity. As can be seen, at the start of the reaction the experimentally measured values were distinctly higher than the value expected in the absence of interaction. Therefore at an actin concentration of 2 mg/ml a marked viscosity increase accompanied the actin activation of the HMM ATPase; and in a similar experiment at 1 mg/ml of actin concentration an almost identical viscosity increase occurred. However, in both cases this increase was small compared to the viscosity increase which occurred after all of the ATP was hydrolyzed to ADP and P_i. In fact, following hydrolysis of the ATP, the samples shown in Figure 3A gelled and the viscosity became unmeasurably large.

To compare the viscosity in the presence and absence of ATP, we studied the viscosity of the actin–HMM mixtures after all of the ATP was hydrolyzed, using very low ratios of HMM to actin so that the viscosity was measurable. The open circles in Figure 4 show the viscosity of such actin–HMM mixtures at three different HMM concentrations and the solid circles show similar viscosity data in the presence of ATP under conditions of nearly maximal actin activation. As can be seen, after all of the ATP is hydrolyzed, it takes only 2% as much HMM to cause the same viscosity in the absence of ATP as it takes in the presence of ATP under conditions of maximal actin activation. Therefore if viscosity were a linear measure of actin–HMM binding, these data would suggest that only about 2% of the HMM is bound to actin under conditions of maximal actin activation.

We also investigated the viscosity of actin–S-1 mixtures in the presence and absence of ATP. As with acto-HMM, in the presence of ATP at 0.1 *M* KCl, the viscosity of the actin–S-1 mixture is equal to the theoretical value expected for completely dissociated acto-S-1 (Figure 3B, open and solid squares). On the other hand, in the absence of KCl under conditions of nearly maximal actin activation there is a clear increase in viscosity above the theoretical value (open and solid circles), although it is not as marked as occurred with HMM under similar conditions. As with acto-HMM, however, the viscosity increased to unmeasurably high values after all of the ATP was hydrolyzed to ADP and P_i. To compare the viscosity of the acto-S-1 in the presence and absence of ATP, we studied the viscosity of the acto-S-1 after all of the ATP was hydrolyzed. As in the similar experiments with acto-HMM, very low ratios of S-1

Table I: Bandwidths, $\bar{\Gamma}$, of Light Scattered from Actin, HMM, and S-1 in the Presence of ATP at 0.1 *M* KCl.[a]

Angle (deg)	q^2 (cm^2)	Fitted Bandwidths (msec^{-1})			
		HMM	S-1	Actin (prep 3)	Actin (prep 7)
63.8	5.0×10^{-10}	2.44 ± 0.39	9.41 ± 0.86	0.583 ± 0.073	0.91 ± 0.17
90	6.7×10^{-10}	4.36 ± 0.52	31.0 ± 1.1	1.07 ± 0.28	1.73 ± 0.12
116.2	1.00×10^{-9}	5.53 ± 0.59	28.7 ± 3.2	1.84 ± 0.21	2.22 ± 0.20

[a] Conditions same as Figure 5.

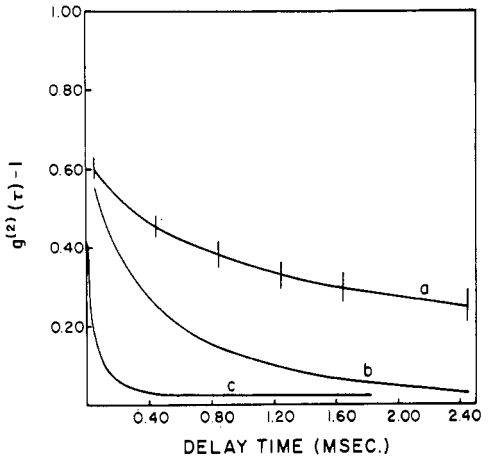

FIGURE 5: Typical intensity fluctuation autocorrelation functions of actin, HMM, and S-1. Conditions: 3 m*M* MgATP; 2 m*M* P_i; 3 m*M* imidazole (pH 7.0); 0.1 *M* KCl; temperature, 0.5°; (a) 1 mg/ml of actin, average trace from several runs using a single preparation; (b) 2 mg/ml of HMM, average trace; (c) 2 mg/ml of S-1 average trace sketched in from data on a different timebase. HMM and S-1 data showed very little scatter and therefore error bars are omitted.

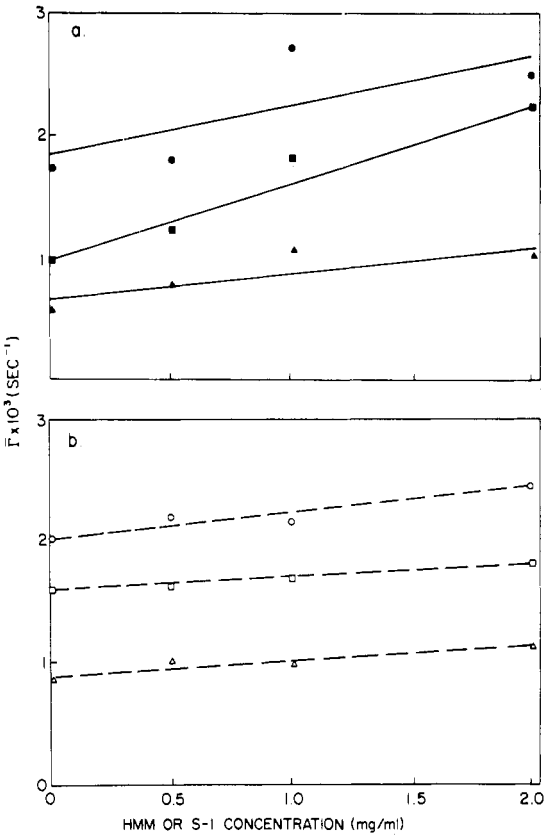

FIGURE 6: Bandwidths, $\bar{\Gamma}$, of acto-HMM and acto-S-1 in the presence of ATP at 0.1 *M* KCl. Conditions same as Figure 5. All preparations contained 1 mg/ml of actin. (a) HMM, (b) S-1. External scattering angle: (●,○) 116.2°; (■,□) 90°; (▲,△) 63.8°.

to actin were used so that the viscosity was measurable. The open squares in Figure 4 show the viscosity after all of the ATP was hydrolyzed and the solid squares, the viscosity in the presence of ATP at nearly maximal actin activation. As can be seen it takes 10% as much S-1 to cause a given viscosity increase in the absence of ATP as is required in the presence of ATP. This is more than was necessary with HMM but nevertheless, as with the acto-HMM, most of the S-1 seems to be dissociated from the actin under conditions of maximal actin activation of the S-1 ATPase.

Intensity Fluctuation Autocorrelation. Repeatable intensity fluctuation autocorrelations for actin, HMM, and S-1 were obtained at scattering angles between 40 and 130°. The data for actin varied somewhat from preparation to preparation and somewhat less from sample to sample in one preparation. Nevertheless all the actin intensity fluctuation autocorrelation data were generally similar and differed dramatically from S-1 and HMM data. Figure 5 shows typical intensity fluctuation autocorrelation functions of the three proteins. The slowly decaying intensity autocorrelation function for the actin preparation indicates that the protein diffused slowly. The nonexponential character of the actin autocorrelation is consistent with a large molecule having hindered diffusion, rotation, and perhaps flexion. HMM and S-1 produced intensity fluctuation autocorrelation functions having rapid decays to the asymptote. This is an indication of the more rapid diffusion of smaller particles. Table I shows the bandwidths, $\bar{\Gamma}$, derived from the data in Figure 5 at 90° as well as similar data at two other scattering angles. To show the variability in the actin, data for two different preparations are given. The large bandwidths for the myosin fragments indicate rapid diffusion, and the approximately linear dependence of $\bar{\Gamma}$ on q^2 indicates that simple translational diffusion dominated. Approximate diffusion constants fitted to the data in Table I are 1×10^{-7} cm^2/sec for the HMM preparation and 6×10^{-7} cm^2/sec for the S-1 preparation. Actin produced nonlinear q^2 plots and therefore we did not estimate a diffusion coefficient, but it plainly diffuses about an order of magnitude more slowly than the HMM and S-1. At 1 g/l. and 90°, actin scattered about three times as much light as HMM, and about an order of magnitude more light than S-1.

When HMM was mixed with actin at 0.1 *M* KCl where little actin activation occurs and the proteins are completely dissociated the resulting intensity autocorrelation functions were more rapidly decaying than they were with only actin present. Accordingly the bandwith $\bar{\Gamma}$ (which is a measure of the rate of decay of the autocorrelation function), increased with increasing HMM concentration at three different scattering angles as shown in Figure 6a. This result was caused

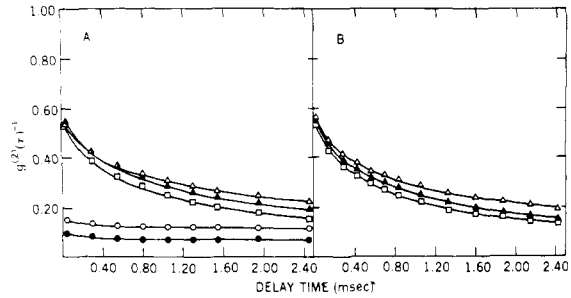

FIGURE 7: Autocorrelation functions of acto-HMM and acto-S-1 as ATP is hydrolyzed. Conditions: 3 m*M* MgATP, 2 m*M* P_i, 2 m*M* imidazole (pH 7.0), temperature, 0.5°; (A) 2 mg/ml of actin and 2 mg/ml of HMM. (B) 1 mg/ml of actin and 1 mg/ml of S-1. Time after start of reaction in A (△) 3.5 min, (▲) 8 min, (□) 13 min, (○) 16 min, (●) 20 min; in B, (△) 6 min, (▲) 12 min, (□) 27 min.

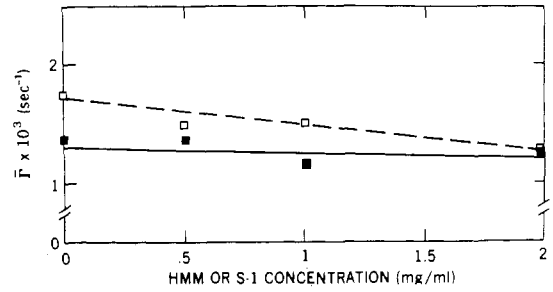

FIGURE 8. Bandwidths, $\bar{\Gamma}$, of acto-HMM and acto-S-1 in the presence of ATP. Conditions: Same as Figure 7 except HMM and S-1 concentrations were varied as shown on the ordinate. Scattering angle = 90° (■) HMM added, (□) S-1 added.

by the more rapidly diffusing HMM contributing a significant amount of more rapid fluctuations to the scattering field. The increasing bandwidth with increasing HMM is consistent with the idea that in the absence of interaction between the actin and HMM the HMM diffuses independently of the F-actin. Figure 6b shows at three different scattering angles the change in bandwidth $\bar{\Gamma}$ of S-1–actin mixtures at 0.1 *M* KCl as the S-1 concentration was increased. Again there was an increase in bandwidth with increasing S-1 concentration but the effect is less marked than with HMM. This is expected qualitatively because S-1 scatters considerably less light than HMM. Nevertheless the data still imply the free diffusion of the S-1 independent of the actin.

Acto-HMM or acto-S-1 systems having nearly maximal actin-activated ATPase hydrolyzed ATP so rapidly that there was only enough time to collect systematic data at one scattering angle and 90° was chosen. The data for acto-HMM is shown in Figure 7a. Intensity autocorrelation measurements were started 1.5 min after the proteins were mixed under the nearly maximal ATPase conditions. Rapid sequential experiments (not shown) demonstrated that the autocorrelation functions were constant for about the first 4 min. Thereafter there was a slight and gradual decrease in the decay rate of the autocorrelation function as shown by the 8- and 13-min curves until the time of ATP exhaustion. At this point large changes in scattered intensity occurred and gel patterns were established as shown by the 16-min curve. The 20-min curve is slightly different than the 16-min curve because during this period the gel became slightly more stabilized and therefore the average scattered intensity became more constant.

Bandwidths, $\bar{\Gamma}$, were not fit to gel autocorrelations because the meaning of the bandwidth is greatly altered by the fact that there was reduced movement of the material, and the field scattered by stationary material inevitably heterodyned with the field scattered by the moving elements. Nevertheless it is clear that following hydrolysis of all of the ATP, the resulting acto-HMM gels had greatly reduced motion, as was indicated by the autocorrelation function showing almost no molecular motion. Therefore we can conclude that the acto-HMM autocorrelation function under conditions of maximal actin activation is markedly different from the autocorrelation function after all of the ATP is hydrolyzed and the HMM is completely bound to actin.

As shown in Figure 7B, the time course data for acto-S-1 is quite different from that of acto-HMM since the final acto-S-1 gel allows full molecular motion at the level probed by the scattered light. This result is consistent with recent work of Abe and Maruyama (1974) who using dynamic viscosity measurements showed marked differences in acto-HMM and acto-S-1 gels. Nevertheless, as shown in Figure 7B, although not as marked as with acto-HMM there is still a clear difference between the pattern after gellation when all of the S-1 is bound to actin, and the pattern when the actin-activated S-1 ATPase is close to its maximum value. Therefore, for both HMM and S-1, the autocorrelation functions suggest that more binding is occurring after all of the ATP is hydrolyzed than at maximum actin activation.

The question then arises whether there is any observable interaction between the actin and HMM in the presence of ATP under conditions of maximal actin activation.

Figure 8 shows the changes in $\bar{\Gamma}$ as a function of S-1 or HMM concentration at an actin concentration of 1 mg/ml where the actin activation is approaching its maximal value. The autocorrelation functions for these systems were nearly identical with those of actin alone, with a slight decrease in bandwidth apparent with increasing HMM or S-1 concentration. This is in contrast to the systems at 0.1 *M* KCl which showed an increase in bandwidth with increasing HMM or S-1 concentration. Therefore these data suggest that some interaction is occurring between the actin and myosin subunits under conditions of maximal actin activation, but much less than occurs after all of the ATP is hydrolyzed.

Discussion

The three different methods used to study the interaction of actin with HMM and S-1 in the presence of ATP gave essentially the same result. There is a marked difference between the physical state of acto-HMM and acto-S-1 in the absence of ATP compared to that in the presence of ATP, even under conditions of nearly maximal actin activation of the HMM or S-1 ATPase. In the presence of ATP, at 0.1 *M* KCl where little actin activation occurs, the viscosity and turbidity are essentially equal to the value expected for no interaction between the proteins; and the quasielastic light scattering data under this condition also suggest that the acto-HMM and acto-S-1 are behaving approximately as an ideal noninteracting system. Both the HMM and S-1 cause an increase in bandwidth and, in the case of HMM, the intensity autocorrelation functions of the mixtures are predictable from the individual components.

On the other hand in the absence of ATP, where ultracentrifuge studies show conclusively that all of the HMM is bound to actin, the viscosity and turbidity measurements re-

flect the presence of a gel and the intensity autocorrelation functions show markedly decreased motion of the actin in the case of acto-S-1 and almost totally bound actin and HMM in the case of acto-HMM. We therefore have marked differences in the viscosity, turbidity, and intensity fluctuation autocorrelation of the totally bound systems which occur in the absence of ATP and the completely noninteracting systems which occur at 0.1 *M* KCl in the presence of ATP.

The question then is how much binding occurs under conditions of maximal actin-activation of the HMM or S-1 ATPase. Qualitatively the viscosity and turbidity measurements suggest that the acto-HMM and acto-S-1 are more than 90% dissociated, but such a conclusion depends on the assumption that viscosity and turbidity are linear measures of binding between actin and the myosin subunits. In fact this assumption does not appear to be quantitatively accurate. It is quite likely that cooperative effects do occur in the viscosity increase caused by the binding of HMM or S-1 to actin. Such cooperative effects may explain why the viscosity increase in the absence of ATP is not linearly related to HMM and S-1 concentration as shown in Figure 4. It may also explain why 10% as much S-1 but only 2.5% as much HMM cause the same viscosity increase in the absence of ATP as in the presence of ATP.

Is it possible then that viscosity and turbidity are not a measure of binding between actin and HMM or S-1 at all, and in fact complete binding can occur in the presence of ATP without any increase in viscosity or turbidity? If this were true, it would mean that unlike acto-HMM which has a very high viscosity and turbidity, acto-HMM-ATP would have the same low viscosity and turbidity as dissociated actin and HMM. This in turn would mean that experiments which depend on turbidity as a measure of the rate or the amount of dissociation of acto-HMM would be invalid and therefore the important conclusion of Lymn and Taylor (1971) that dissociation of acto-HMM occurs more rapidly than the initial burst of ATP hydrolysis would also be in doubt.

In fact, however, although viscosity and turbidity may not be linear measures of binding between actin and HMM, their low values under conditions of maximal actin activation probably do reflect dissociation of the acto-HMM and acto-S-1 since other measurements such as spin-label studies (Seidel, 1973; Stone, 1973) as well as the more direct ultracentrifuge and kinetic studies (Eisenberg et al., 1972; Eisenberg and Kielley, 1972) also suggest marked dissociation is occurring under these conditions. It therefore seems likely that at least some of the difference in the viscosity and turbidity of acto-HMM and acto-S-1 in the presence and absence of ATP is due to dissociation of actin and myosin subunits under conditions of maximal actin activation. Quantitatively, however, the viscosity and turbidity data are difficult to interpret.

Unfortunately quantitative interpretation of the intensity fluctuation autocorrelation data is also difficult. The weighting in these experiments favors the larger molecules, as is shown by actin scattering per unit weight three times as much light as HMM and an order of magnitude more than S-1. Therefore we are looking at the motion of the actin much more than the motion of the HMM or S-1. Nevertheless the smaller myosin fragments did appreciably increase the bandwidth of the scattered light in the noninteracting systems at 0.1 *M* KCl. On the other hand at nearly maximal actin activation, the acto-HMM and acto-S-1 produced intensity autocorrelation functions that differed little from those of action alone. This occurred even though the viscosity of the system had increased significantly—nearly double in the case of actin at 1 mg/ml and HMM at 2 mg/ml. The increased viscosity implies decreased rates of relatively large scale motion of the molecular system in shear fields, but nearly unchanged rates of microscopic motions of the molecules as weighted by their scattering powers. The simplest interpretation of these data is that the interacting systems had most of their myosin fragments free to diffuse, and this effect cancelled out the decrease in bandwidth caused by slowed diffusion of a small amount of actin complexed with HMM. Of course as in the case of viscosity and turbidity other explanations are not yet eliminated, such as that the myosin fragments all bind and the actin–myosin system becomes more viscous in flow, but some increased flexibility in the actin polymers permits diffusive internal motions that account for compensating fluctuations.

Summing up all of our data then, there is a marked difference in the viscosity, turbidity, and quasielastic light scattering of acto-HMM and acto-S-1 in the absence of ATP and in the presence of ATP under conditions of maximal actin activation. This difference suggests but does not prove that even at maximal actin activation of the HMM and S-1 ATPase most of the HMM and S-1 are dissociated from actin and are therefore in the refractory state unable to bind to actin, while only a small fraction at any one time is in the nonrefractory state complexed with actin.

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The Complete Amino Acid Sequence of Ubiquitin, an Adenylate Cyclase Stimulating Polypeptide Probably Universal in Living Cells†

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ABSTRACT: The complete amino acid sequence was determined for bovine ubiquitin, an adenylate cyclase stimulating polypeptide, which is probably represented universally in living cells. Ubiquitin has a molecular weight of 8451 and consists of a single polypeptide chain containing 74 amino acid residues. It contains four arginine residues but no cysteine or trytophan residues. The first 61 amino acid residues were obtained by automated Edman degradations. Tryptic digestion of maleated ubiquitin yielded four peptide fragments that were resolved by molecular sieve chromatography and coded in order of decreasing chain length (MT-1, MT-2, MT-3, and MT-4). The automated sequenator determinations on native ubiquitin provided overlapping sequence data for three of these fragments that gave an order of MT-1, MT-3, and then MT-2. Peptide MT-4, a dipeptide, was therefore assigned to the C terminus, and the placement of peptide MT-2 was corroborated by analysis of data from carboxypeptidase digestions of maleated ubiquitin. Peptide MT-2 was demaleated and sequenced by manual Edman degradations through a single lysine residue. it was cleaved at this residue with trypsin, and the two resultant peptides were separated by ion-exchange chromatography. Manual sequencing of the C-terminal demaleated tryptic peptide of MT-2 completed the sequence of MT-2 and that of native ubiquitin. The sequence of ubiquitin was further confirmed and supported by amino acid and partial sequence analysis of fragments obtained by digestion of maleated ubiquitin with chymotrypsin or staphylococcal protease.

Ubiquitin (formerly ubiquitous immunopoietic polypeptide or UBIP) is an 8451-dalton polypeptide first isolated from bovine thymus but subsequently found to be present in the cells of all tissues studied and, indeed, in the cells of most living organisms, as judged from examples of animals, yeast, bacteria, and higher plants (Goldstein et al., 1975). Ubiquitin was purified in the course of isolation of thymopoietin, a polypeptide hormone of the thymus (Goldstein, 1974). Thymopoietin specifically induces the differentiation of thymocyte precursors, presumably by combining with a receptor on the prothymocyte membrane and triggering adenylate cyclase activation (Basch and Goldstein, 1974; Scheid et al., 1975). Ubiquitin can mimic thymopoietin-induced differentiation, but its action is not restricted to prothymocytes, since it can activate B-cell differentiation (Goldstein et al., 1975; Scheid et al., 1975) and adenylate cyclase in a wide variety of tissues (Bitensky and Goldstein, 1975). The stimulation of adenylate cyclase by ubiquitin is inhibited by propranolol (Goldstein et al., 1975; Scheid et al., 1975), and it is therefore presumed to have a β-adrenomimetic active site and to act via a β-adrenergic receptor. Ubiquitin shows a high degree of evolutionary conservation, exhibiting close functional and immunological similarity when isolated from such diverse origins as cells of mammals and higher plants (Goldstein et al., 1975). In a preliminary study comparing the first eight amino acid residues of the N-terminal sequence of bovine and celery ubiquitin, there were six identical residues and one substitution at position 1; position 4 of celery ubiquitin was not identified.

The exact function of this adenylate cyclase stimulating polypeptide remains unknown; that it subserves some function vital in the living organism may be inferred from its extraordinary evolutionary conservation. The likely structural

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