*STUDIES ON THE STRUCTURAL BASIS OF THE INTERACTION OF MYOSIN AND ACTIN**

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From the extensive electron microscopic studies by Huxley,[1] we now have a detailed picture of the filament structure of striated muscle. These studies indicate that the basic functional unit of the sarcomere consists of two kinds of mutually parallel filaments. One of these filaments (the thick filament) is composed of a closely packed array of myosin molecules, and the other (the thin filament) is composed of fibrous actin. These two structures are connected at regular intervals along the filament axis by cross-bridges which emerge as lateral projections from the myosin-containing filament. Each of these cross-bridge segments is now believed to represent (at least in part) the ATPase-active heavy meromyosin (HMM) region of the myosin molecule.[1] The actin filament consists of two helical strands, and each strand is composed of identical repeating units of globular actin.[2]

Studies on the structure of myosin in solution have led to the idea that the HMM region of this particle displays three separately folded (and presumably identical) polypeptide chains, which may be dissociated from one another by brief tryptic digestion of HMM.[3] Recently, this view has received support from the finding that both myosin and HMM possess three apparently identical binding sites for ADP, whereas the smaller ATPase-active tryptic fragment of heavy meromyosin (termed subfragment I by Mueller and Perry[4]) binds only one molecule of ADP per particle.[5] We have interpreted this result to mean that these nucleotide-binding sites are the catalytic sites of myosin and that each one is located within one of the globular "appendages" (subfragment I) which have been proposed to comprise the heavy meromyosin region of the parent molecule.[5]

In the study presented below, we have measured the binding of both myosin and subfragment I by fibrous actin in solution: (1) to determine the number of actin monomer units within the F-actin double helix which specifically interact with a single HMM particle; and (2) to see whether each of the proteolytic fragments of HMM (subfragment I) is functionally capable of interacting with F-actin, or whether only one of them exhibits this property.

Experimental.—Reagents and preparation of the myosin fragments: Doubly glass-distilled water was used for all solutions. Rabbit skeletal myosin was prepared as previously described.[6] Heavy meromyosin was isolated and purified from 75-sec tryptic digests of myosin under the conditions described by Young *et al.*[7] This digestion procedure minimizes proteolytic degradation of HMM and yields a highly homogeneous preparation of this protein.

Subfragment I was prepared as follows. Solutions of HMM isolated as described above (30 mg/ml in 0.05 M KCl, 0.1 M tris-HCl, pH 7.6) were treated at 25° with 2 mg/ml of 2× recrystallized trypsin (Worthington Biochemicals). After 5–40 min of proteolysis, the reaction was stopped by addition of soybean trypsin inhibitor (Worthington, 3× recrystallized) to a final concentration of 4 mg/ml. Subfragment I was isolated from these solutions by Sephadex G-200 chromatography as previously described.[3] Preparations of subfragment I isolated in this way exhibit a high degree of electrophoretic[8] and weight-class[3, 8] homogeneity. Moreover, the physical and chemical properties of this macromolecule are relatively insensitive to the time of tryptic digestion used to liberate it from HMM.[8]

Preparation of globular and fibrous actin: G-actin was isolated from acetone powders of rabbit skeletal muscle and purified by gel-filtration chromatography (Sephadex G-200) as described by Rees and Young.[9] This procedure yields electrophoretically homogeneous solutions of G-actin of uniform particle mass (mol wt = 46,000 gm/mole), and these preparations are quantitatively converted to high-molecular-weight fibrous actin in the presence of 0.1 M KCl, 1 mM $MgCl_2$.[9] Free, unbound bulk solvent ATP was removed from solutions of F-actin as follows. Dowex-3 (100–200 mesh) was washed successively with 0.5 N NaOH and 0.5 N HCl and equilibrated with 0.1 M KCl, 1 mM $MgCl_2$, 0.01 M tris-HCl, pH 7.6. To a 0.8 × 3-cm column of this resin 10–15 ml of a 1–2 mg/ml solution of F-actin prepared as described above was applied. The protein passed readily through the column and the free nucleotide was quantitatively retained. Of particular importance is the fact that this procedure did not cause partial depolymerization of F-actin to lower molecular weight or inactive components (see below).

Physical measurements: Concentrations of solutions of myosin, HMM, and subfragment I were determined spectrophotometrically (λ = 280 mμ), together with respective extinction coefficients of 0.540, 0.647, and 0.770 ml/mg-cm.[3] The concentration of G-actin was determined both by the Biuret method standardized against multiple microkjeldahl analyses and by spectrophotometric measurements. The specific extinction coefficient of G-actin was taken to be 1.09 ml/mg-cm (λ = 280 mμ).[9]

A Beckman model E analytical ultracentrifuge equipped with an electronic speed control, monochromator, and photoelectric scanning absorption optics was used to study the binding of HMM and subfragment I by F-actin. The interference optical system of the instrument was aligned by the procedures of Richards and Schachman[10] and the absorption optical system by the techniques of Schachman *et al.*[11] To minimize stray light striking the photomultiplier, radially oriented masks (4 × 20 mm) were mounted over the collimating and condensing lenses of the absorption optical system. Under these conditions, optical density was a linear function of protein concentration ($0 < OD < 1.4$; λ 280 mμ). Two double-sector aluminum-Epon cells with sapphire windows were used for all measurements. These cells were carefully selected so that when both sectors were filled with water, the scanner recorded exactly zero absorbance throughout the cell at the highest rotor velocities employed (rpm = 60,000 rpm). Photographic plates (Eastman Spectroscopic, type IIG) were analyzed with a Nikon model 6 microcomparator.

Results.—To measure the binding of HMM and subfragment I by fibrous actin at chemical equilibrium, we have taken advantage of the very high molecular weight and sedimentation rate of F-actin compared to that of the myosin component. Let us consider an HMM solution to which has been added a small amount of F-actin. Since the double-stranded actin polymer is assembled from a large number of monomer units, several particles of HMM will be bound by each actin chain; and, as we increase the total concentration of HMM, progressively more and more of the actin monomer sites will be occupied. If these mixtures are now centrifuged at high speed, the HMM-F-actin complex will migrate rapidly to the base of the centrifuge cell and the supernatant solution will contain free, unbound meromyosin. Furthermore, if the system is at chemical equilibrium prior to centrifugation, and if this is not altered by the transport process itself, then the true equilibrium concentration of HMM will be given by the measured concentration after the HMM-actin complex has migrated to the bottom of the cell. (See ref. 12 for a discussion of this point.) An experiment of this type is thus analogous to one in which the binding of a small organic molecule to protein is evaluated from its transport in the presence and absence of the more rapidly sedimenting macromolecule (see, for example, ref. 13).

For the binding studies, a series of solutions was prepared which contained constant concentrations of HMM (or subfragment I) in the presence of increasing concentrations of F-actin. Each of these solutions was placed in one limb of a double-sector capillary synthetic boundary cell (when refractometric optics were used) or a

standard double-sector cell (when absorption optics were used). Solvent was added to the other channel and the rotor was rapidly accelerated to 40,000–60,000 rpm. Photographs or scanner traces were taken within five to ten minutes after this speed had been reached. Within this time period, the F-actin complex had migrated to the bottom of the cell and only a single boundary due to the free, unbound myosin component remained (see Fig. 1). At the higher initial concentrations of the myosin component (5 mg/ml), the refractometric optical system was used to measure the free concentration of this component. At lower levels of protein concentration (1–2 mg/ml), absorption optics were employed.

Figure 1 presents a series of refractometric optical patterns of HMM as a function of the total F-actin concentration (0.26–1.19 mg/ml). The free concentration of HMM in each solution was computed both from schlieren area measurements and from the number of interference fringes across the solvent-solution boundary. These diagrams reveal that as the F-actin concentration increases, the concentration of free HMM decreases. Figure 2 illustrates photoelectric scanner traces

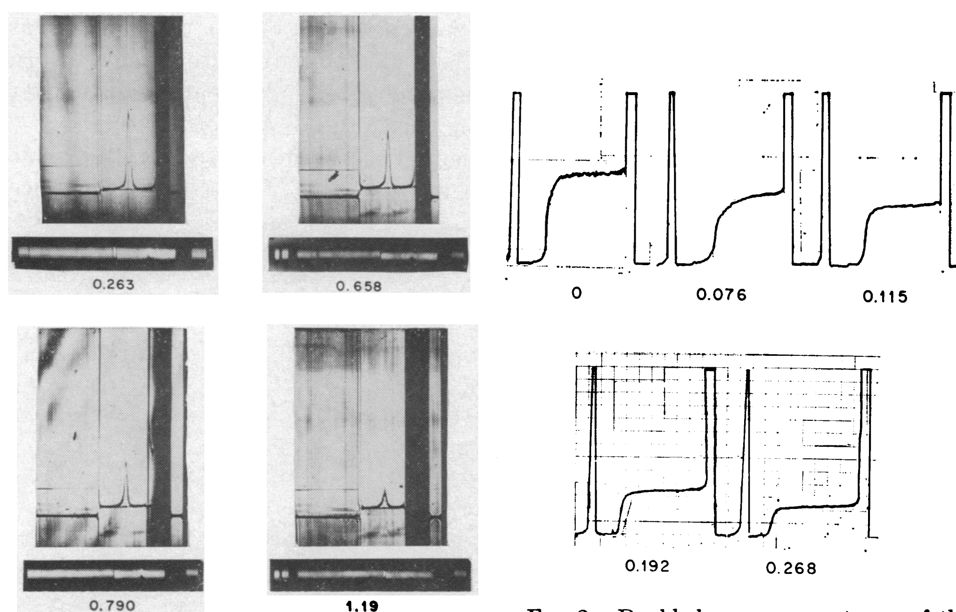


FIG. 1.—Refractometric optical patterns of the binding of HMM by F-actin. Increasing quantities of F-actin (1.58 mg/ml in 0.1 *M* KCl, 1 mM $MgCl_2$, 0.01 *M* tris-HCl, pH 7.6) were added to a solution containing 5.94 mg of HMM dissolved in 0.05 *M* KCl, 0.1 *M* tris-HCl, pH 7.6. The total volume of each solution was adjusted to 1.2 ml with the F-actin solvent (0.1 *M* KCl, 1 mM $MgCl_2$, 0.01 *M* tris-HCl, pH 7.6). The total HMM concentration was 4.95 mg/ml and the total F-actin concentration (mg/ml) in each solution is given in the figure. Schlieren and interference photographs were taken 10 min after reaching a speed of 42,040 rpm and the boundaries shown represent those corresponding to free, unbound HMM. Temperature: 6–8°.

FIG. 2.—Double-beam scanner traces of the binding of HMM by F-actin. Aliquots of an F-actin solution (c = 1.5 mg/ml in 0.1 *M* KCl, 1 mM $MgCl_2$, 0.01 *M* tris-HCl, pH 7.6) were added to 4.3 mg of HMM (solvent: 0.05 *M* KCl, 0.1 *M* tris-HCl, pH 7.6). The volume of each solution was adjusted to 2.0 ml with the F-actin solvent. Final F-actin concentrations (mg/ml) are given in the figure and the total HMM concentration was 2.15 mg/ml for all samples. Photoelectric scanner traces were taken 10–30 min after reaching a rotor speed of 60,000 rpm. Direction of sedimentation is to the right and each centimeter on the ordinate represents 0.4 OD units. The monochromator was set at λ = 280 mμ and the photomultiplier slit width was adjusted to 0.16 mm at the plane of the image. Temperature: 6–8°.

(λ = 280 mμ) from a study similar to that shown in Figure 1. In this case, however, the total initial concentration of reactants was reduced approximately 2.5-fold. (The direction of sedimentation is to the right and optical density is proportional to vertical distance along the ordinate.) Again we see that the concentration of free HMM decreases as a function of increasing F-actin concentration. The scanner traces also demonstrate that the concentration of HMM is uniform in the "plateau" region. This feature indicates that serious re-equilibration effects are probably absent during the transport of F-actin-HMM complexes across the solution column. It should be noted that the results presented in Figures 1 and 2 were independent of the time at which the HMM-actin solutions were analyzed after mixing (1–24 hr).

To establish that all of the G-actin preparations were quantitatively transformed to F-actin, each sample was examined with the photoelectric scanner. In agreement with previous studies on the polymerization of chromatographically isolated G-actin, all F-actin solutions exhibited only a single, rapidly migrating boundary.[9] To ensure that the sedimentation boundaries shown in Figures 1 and 2 actually represent only free HMM, the sedimentation coefficient of this component was measured after the actin-HMM complex had sedimented to the base of the cell. In a typical case, $s_{20,w}$ was found to be 6.55—a value essentially identical to that for the same concentration of HMM (c = 4.3 mg/ml) in the absence of F-actin ($s_{20,w}$ = 6.6S). Similarly, the sedimentation coefficient of subfragment I sedimenting alone (c = 0.64 mg/ml) was $s_{20,w}$ = 5.72S as compared to a value of 5.56S in the presence of F-actin.

In Figure 3, the free, unbound concentrations of both HMM and subfragment I,

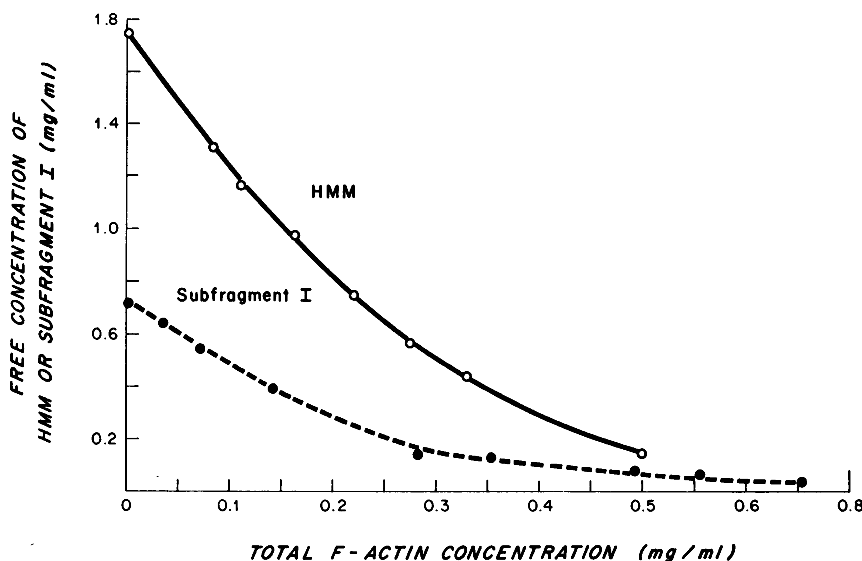


FIG. 3.—Concentrations of free heavy meromyosin and subfragment I as a function of the total F-actin concentration. Concentrations of the free myosin components were evaluated from scanner traces such as those presented in Fig. 2. The HMM and subfragment I solvents contained 0.04 M KCl, 0.4 mM $MgCl_2$, 0.07 M tris-HCl, pH 7.6 and 0.075 M KCl, 0.5 mM $MgCl_2$, 0.05 M tris-HCl, pH 7.6, respectively. Temperature: 6–8°.

TABLE 1

Summary of Calculated Values of ν_{max} and k_{int} for the Binding of HMM and Subfragment I by F-Actin

Preparation HMM	Total concentration (mg/ml)	Time of tryptic digestion (min)	Rotor velocity	k_{int} (M)	ν_{max}
9	4.95	—	42,040	3.4×10^{-6}	1.1
12	3.01	—	40,000	3.0×10^{-6}	1.2
13	2.24	—	40,000	1.4×10^{-6}	1.0
37	2.15	—	60,000	1.6×10^{-6}	1.1
				Mean:	1.1
Subfragment I:					
11	2.91	5	42,040	1×10^{-6}	0.9
24	0.70	20	40,000	1×10^{-6}	1.2
				Mean:	1.0

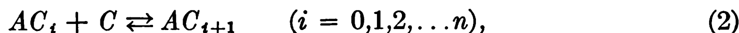
Time of tryptic digestion refers to the time interval used to prepare subfragment I by tryptic proteolysis of HMM as discussed in the *Experimental* section. Values of k_{int} and ν_{max} (moles HMM [or subfragment I] bound per mole of actin monomer) were computed from data such as that presented in Fig. 4. Temperature: 6–8°.

derived from data such as that in Figures 1 and 2, are plotted as a function of the total F-actin concentration. These data demonstrate that both HMM *and subfragment I* are bound quantitatively as the actin complex in the presence of sufficiently high concentrations of F-actin. Thus, in agreement with earlier evidence for the chemical homogeneity of subfragment I,[8] the present studies indicate that each of these tryptic fragments of HMM possesses a specific actin-binding site.

The plots illustrated in Figure 3 closely obey a simple adsorption isotherm over a wide range of protein concentrations (see Table 1). Consequently, these data have been analyzed according to the theory of multiple equilibria and equation (1).[14, 15]

$$\nu/c = (\nu_{max} - \nu)k_{int}^{-1}. \quad (1)$$

This equation has been formulated on the basis of set of simultaneous equilibria of the kind:



$$AC_i + C \rightleftarrows AC_{i+1} \qquad (i = 0,1,2,\ldots n), \quad (2)$$

where C is the molar concentration of free, unbound myosin component. The AC_i represent molar concentrations of the various molecular complexes of C with A (F-actin), and k_{int} is the apparent intrinsic dissociation constant of a single actin monomer-myosin component complex. Values of ν have been calculated to represent the average number of moles of HMM bound per total moles of actin *monomer*. Thus,

$$\nu = \frac{\sum_{i=1}^{n} iAC_i}{G_0} = \frac{C_0 - C}{G_0}, \quad (3)$$

where G_0 is the total molar concentration of actin monomer, and C_0 is the total concentration of the myosin component. To compute ν, the molecular weights of HMM, subfragment I, and G-actin were taken to be 380,000,[3] 115,000,[3, 8] and 46,000[9] gm/mole, respectively.

Figure 4 illustrates plots of ν/c versus ν for HMM and subfragment I, respectively, and values of ν_{max} were obtained by extrapolation of these plots to $\nu/c = 0$.

Table 1 summarizes values of k_{int} and ν_{max} obtained from analyses such as that shown in Figure 4 over a wide range of initial concentration of reactants. The data indicate that one actin monomer binds maximally one molecule of HMM *or* one molecule of subfragment I. Table 1 also reveals that the interaction of subfragment I with F-actin is independent of the time of tryptic digestion used to isolate this fragment from HMM.

Discussion.—Earlier studies on the chemical and physical properties of subfragment I have suggested that these particles probably possess identical chemical structures.[5, 8] The present results support this view since they indicate that each of these ATPase-active fragments of HMM also possesses a binding site for F-actin. From their studies, Jones and Perry have also observed that subfragment I may be quantitatively bound as the F-actin complex[16] (see ref. 17).

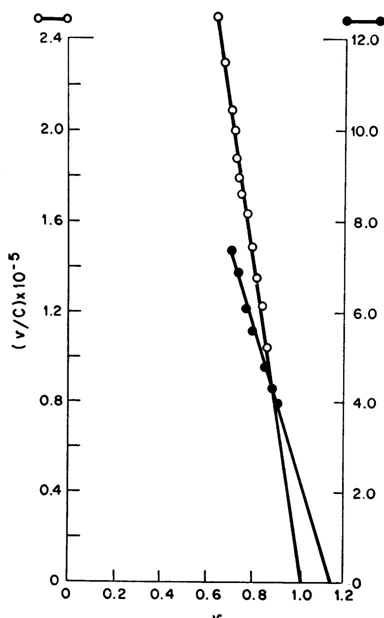


FIG. 4.—Analyses of the interaction of HMM and subfragment I according to equation (1). Values for ν were obtained from data such as that presented in Fig. 3. Left ordinate refers to HMM (○—○); right ordinate, to subfragment I (●—●).

Estimation of stoichiometric combining ratios for actin and myosin is a difficult problem which has received considerable attention. Light scattering and transport studies (see, for example, refs. 18–20) have indicated that one gram of F-actin maximally binds between 2.5 and 4.0 gm of myosin. If we take the molecular weights of myosin and actin to be 600,000[21, 22] and 46,000 gm/mole, respectively, this means that one myosin molecule interacts simultaneously with 3–5 actin monomer units. On the other hand, it should be noted that there are difficulties associated with light scattering measurements on a multicomponent system such as actomyosin. For example, it is not generally possible to estimate directly the free concentration of any of the reactants. Consequently, evaluation of stoichiometric combining ratios involving two or more macromolecules requires assumptions about the way in which the scattering intensity changes as a function of the degree of saturation. Since both myosin and F-actin are highly asymmetric proteins which interact strongly with each other, it seems likely that the scattering intensity will

be a complex function of the degree of saturation. A second problem stems from the recent observation that actin preparations isolated by conventional polymerization-depolymerization procedures contain significant amounts of impurities,[9] and it is possible that this factor is reflected by earlier estimates of the combining ratio. The results presented above were obtained with highly homogeneous preparations of globular actin which were quantitatively transformed to fibrous actin. They indicate that each actin monomer interacts with one HMM particle or with one molecule of subfragment I. Thus, although these findings are not in accord with the studies referred to above,[18-20] they are in excellent agreement with the electron microscopic results of Huxley, who has also inferred that one HMM attaches to one actin monomer unit.[1]

Several lines of evidence now indicate that the globular HMM region of the myosin molecule is probably assembled from multiple polypeptide chains—each of which is potentially able to hydrolyze ATP and each of which is potentially able to combine with one actin monomer of the F-actin double helix. (Determination of the number of individual chains which comprise HMM is a difficult problem, and the answer to it remains uncertain. Some studies favor a 2-chain structure,[23] whereas we believe that this meromyosin is 3-stranded.[3, 5, 8]) Yet the present results indicate that one (multistranded) HMM particle also interacts with one actin monomer unit. If these ideas are correct, they suggest that only one of the polypeptide chains of HMM interacts with a single actin monomer at a time. Several sources of information, both from studies in solution[3] and in the solid state,[24, 25] have indicated that the interfilament cross-bridges are flexible. Thus, it seems possible that small changes in orientation of the two or three chains of HMM relative to each actin monomer could be responsible for movement of the thin filament during contraction.

Summary—The interaction of fibrous actin with the ATPase-active fragments of myosin (heavy meromyosin and subfragment I) has been studied by transport measurements in solution. The results indicate that each monomer unit of F-actin binds one particle of either of these fragments. Since HMM appears to be assembled from more than one identical subfragment I chain, and since each of these fragments possesses an actin-binding site, we infer that only one of them is responsible for linking HMM to the actin chain at a time. Thus, it may be that the sliding of the thin filament during contraction is related to small conformational movements of the globular appendages of HMM relative to one another.

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Abbreviations used: HMM, heavy meromyosin; ADP, adenosine diphosphate; ATPase, adenosine triphosphatase.

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[12] Transport measurements of the kind used in this study are not free from possible complications due to (*a*) interacting flows; (*b*) disturbances of chemical equilibrium which arise from different sedimentation rates of the various molecular complexes; and (*c*) radial dilution of the unbound "ligand" (e.g., HMM or subfragment I). In spite of these potential problems, there are several reasons for believing that re-equilibration effects during the transport process are probably negligible. Radial dilution effects due to sedimentation of the myosin component are minimal since the concentration of this material was measured as soon as possible after the actin-complex had migrated to the bottom of the cell (within 5–10 min at 60,000 rpm). Although the G-actin preparations used in this study are highly homogeneous, the length (and hence mass) of the F-actin chains are undoubtedly not uniform. Thus they must sediment at different rates. However, on a macroscopic scale, only one rapidly migrating boundary ($s_{20,w} \simeq 100S$) was detected at all concentrations of the free myosin component. (This complex boundary may be seen in Fig. 1 (lower left) as a hypersharp refractive index gradient close to the bottom of the cell.) In view of the fact that the concentration of the free myosin component is uniform across the cell (Fig. 2) and that the complex boundary appears uniform and hypersharp, we assume that re-equilibration effects have not significantly affected the data. The fact that similar values of ν_{max} and k_{int} were obtained over a three- to fourfold range in protein concentration supports these ideas (see Table 1). Another potential difficulty arises from the theoretical calculations of Kegeles, Rhodes, and Bethune (these PROCEEDINGS, **58**, 45 (1967)) on the effects of pressure on interacting systems in the ultracentrifuge. The fact that the present results were essentially independent both of protein concentration and of rotor velocity over a twofold range of field strength suggests that pressure effects probably played a minor role.

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