A Solid-Liquid Biphasic Model for Characterization of Properties of Muscle and Platelet Contractile Proteins*

SAUL PUSZKIN,† SHAUL KOCHWA, ELENA G. PUSZKIN, AND RICHARD E. ROSENFIELD

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SUMMARY

Actomyosin, myosin, and actin from different sources are adsorbed, apparently as a monolayer, by polystyrene particles (Lytron 615). The affinity binding constants of these proteins for 1 mg of Lytron were about 10^7 liters mol^-1, while heterogeneity indices (α) varied from 0.70 to 1.0 presumably as a function of spontaneous aggregation in the liquid phase. Adsorption was irreversible. Orientation of adsorbed molecules permitted association of bound muscle myosin with muscle actin and of bound muscle actin with platelet or muscle myosin. The association constant of the former reaction was 2.78 x 10^6 liters mol^-1. Enzymatic properties of adsorbed platelet or muscle contractile proteins remained unchanged. When actin was dissociated from adsorbed actomyosin, Mg^2+ATPase activity was abolished, but association of myosin with bound actin, or association of actin with bound myosin was accompanied by restoration of Mg^2+ATPase activity. Every subunit of F-actin strands, unless F-actin had been fully depolymerized to G-actin, could bind myosin and activate Mg^2+ATPase activity. Immunogenic characteristics of muscle myosin were enhanced by Lytron adsorption. Elicited antibodies showed selective specificity for an antigenic determinant located near or at the actin combining site of muscle myosin. Antibodies did not react with actomyosin. Antibodies prevented association of actin with muscle myosin because they inhibited both superprecipitation and development of Mg^2+ATPase activity.

In the present study, fibrillar contractile proteins were found to bind to Lytron particles. Furthermore, the bound protein retained its specific biochemical activity. This allowed kinetic studies of association-dissociation of myosin-actin systems when only one component was adsorbed by Lytron particles and the other was in solution. Lytron particle bound muscle myosin was highly immunogenic in rabbits, and the elicited antibodies displayed unusual specificity.

EXPERIMENTAL PROCEDURE

Materials—Polystyrene (Lytron) particles (uniform spherical) are prepared commercially by catalytic emulsion polymerization of styrene in the presence of a surface active agent (9). A single batch of Lytron 615, kindly supplied by Dr. E. Rossen (Monsanto Chemical Co., Springfield, Mass.) was used. The dimensions of the particles were 2,500 A diameter and 1,964 X 10^6 A^2 surface. There were 1.19 X 10^15 Lytron particles in 1 mg of dried material. Lytron particles were sedimented from original suspension at 40,000 X g (RC2-B refrigerated centrifuge, Sorvall Co., Norwalk, Conn.) for 30 min, washed, and resuspended in an appropriate buffer at 20 mg per ml. These suspensions remained stable in a variety of buffers containing up to 0.6 M KCl. ATP was purchased from Sigma. Grade II was used for purification of proteins and Grade I for ATPase determinations. Bovine serum albumin was from Lot 16-BU-0162 from Pentex.

Protein Preparations—Muscle actomyosin (10), myosin (11), and actin (13) were prepared from fresh rabbit back muscle and from dog heart and back muscle. Platelet actomyosin, from human blood platelets collected and concentrated by Latham bowl plateletpheresis, was purified by the method of Bettez-Gainard and Lasche (15). Platelet myosin was isolated as described elsewhere (14). Except for immunological studies, all experiments were performed with rabbit back muscle and human platelet contractile proteins.

Unless specified, the following buffers were used: 0.05 M Tris-HCl (pH 7.2), 0.5 M KCl for actomyosin; 0.05 M Tris-HCl (pH 7.2), 0.37 M KCl for myosin; and 0.2 mM ATP, 0.2 mM ascorbate (pH 6.8) for actin. Dithiothreitol, 0.1 mM, was used in all steps of protein preparations (15).

Protein concentration was measured in duplicate samples by the method of Lowry et al. (16). Bovine serum albumin dissolved in 0.1 M KCl or 0.6 M KCl 0.05 M Tris-HCl (pH 7.2) was used as standard.

Polyacrylamide Gel Electrophoresis—Gel electrophoresis was performed at room temperature (Buchler Instruments, Inc., Fort Lee, N. J.) on single 5% polyacrylamide gels containing 7.8 g of NaH2PO4. H2O, 38.6 g of Na2HPO4.7H2O, and 1 g of sodium dodecyl sulfate per liter. Proteins were dissolved in appropriate buffers at concentrations of 2 to 5 mg per ml. Samples containing 75 µg of protein were dissolved in 0.05 ml of 0.01 M phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate, bromphenol blue (tracking dye), and 0.1% 2-mercaptoethanol; the samples were

Polystyrene (Lytron) particles adsorb γG-globulins (1, 2) and IgG-coated Latex suspensions have been used both in agglutinations tests to detect Rheumatoid Factor (3) and a number of serological tests (4–8) involving reactions between adsorbed antibodies and specific antigens. Human IgG adsorbed by Lytron particles is considerably more immunogenic in rabbits than in human IgG in solution (2).

* This study was supported by Grants AM-12912, HD-03972, HL-05488, HL-12443 and AA316 from the National Institutes of Health. This paper is the second of a series on adsorption of proteins for 1 mg of Lytron were about 10^7 liters mol^-1, while heterogeneity indices (α) varied from 0.70 to 1.0 presumably as a function of spontaneous aggregation in the liquid phase. Adsorption was irreversible. Orientation of adsorbed molecules permitted association of bound muscle myosin with muscle actin and of bound muscle actin with platelet or muscle myosin. The association constant of the former reaction was 2.78 x 10^6 liters mol^-1. Enzymatic properties of adsorbed platelet or muscle contractile proteins remained unchanged. When actin was dissociated from adsorbed actomyosin, Mg^2+ATPase activity was abolished, but association of myosin with bound actin, or association of actin with bound myosin was accompanied by restoration of Mg^2+ATPase activity. Every subunit of F-actin strands, unless F-actin had been fully depolymerized to G-actin, could bind myosin and activate Mg^2+ATPase activity. Immunogenic characteristics of muscle myosin were enhanced by Lytron adsorption. Elicited antibodies showed selective specificity for an antigenic determinant located near or at the actin combining site of muscle myosin. Antibodies did not react with actomyosin. Antibodies prevented association of actin with muscle myosin because they inhibited both superprecipitation and development of Mg^2+ATPase activity.

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FIG. 1. a, sodium dodecyl sulfate polyacrylamide gel electrophoresis. A, back muscle myosin; B, platelet myosin; C, back muscle actomyosin; D, platelet actomyosin; E, muscle actin from a separate electrophoretic run. The number of bands observed in C and D represent components of the actomyosin molecules dissociated by the effect of sodium dodecyl sulfate. The second darkest band, situated in the middle of Gels C, D, and E, represents the actin component (E is from a separate run of a muscle actin incubated at room temperature for 16 hours and then placed on top of the separating gel. A current of 1 ma per gel was applied for 45 min to concentrate proteins at the gel entry surface. This was followed by a current of 10 ma per gel until the tracking dye reached the bottom. Gels were stained for 45 min with 1% Amido black 10B in 7% acetic acid and destained by diffusion overnight in 7% acetic acid. Stained gels were scanned at 550 nm with a Gilford densitometer attached to an automatic Gilford recording spectrophotometer (Gilford Instruments, Oberlin, Ohio).

* The relative concentration of bands was measured with an integrator attached to a PDP 11 computer (Digital Equipment Co., Maynard, Mass.). Contaminants represented 20.2% for platelet actomyosin and 16.6% for platelet myosin. Less than 5% contaminants were found in muscle preparations (Fig. 1, a and b).

Coating of Lytron Particles with Fibrillar Proteins—Protein concentrations were adjusted to approximately 1.2 mg per ml. One volume of Lytron particle suspension, 20 mg per ml of water, was added to 9 volumes of protein solution in appropriate buffer, and the mixture was gently agitated at room temperature. Adsorption was complete within 3 to 5 min. Protein-coated Lytron particles were sedimented at 10,000 x g at 4° for 10 min, and the pellet was washed once with appropriate buffer.

The amount of protein adsorbed was estimated from the difference between the initial protein concentration in solution and the remainder after adsorption. For determination of enzymatic activity, Lytron particles were resuspended in appropriate buffer and concentration was adjusted so that final suspensions contained 1 mg of bound platelet protein per ml or 0.1 mg of bound muscle protein per ml.

Determination of ATPase Activity—ATPase activity of platelet and muscle proteins was determined by the release of inorganic phosphorus (P_i) from ATP according to Marsh (17) but modified to detect 3 nmol of P_i (15). One-tenth-milliliter-volumes of protein samples, in solution or Lytron particle-bound, were added to 0.8 ml of 1 mM Mg^2+ or 1 mM Ca^2+. For assay purposes the quantity of protein was 0.01 mg for muscle actomyosin and muscle myosin, and 0.1 mg for platelet actomyosin and platelet myosin. Muscle actin (0.02 mg) was used for activation of Mg^2+ATPase activity; the reaction was initiated by addition of 0.1 ml of 5 mM ATP, and allowed to proceed at 25° for 15 min for muscle proteins and 30 min for platelet proteins. Reactions were stopped by addition of 0.4 ml 20% trichloroacetic acid. Blanks consisted of proteins trichloroacetic acid precipitated prior to addition of ATP. ATPase activity was estimated as the difference between P_i at the end of the reaction and P_i at zero time.

Superprecipitation—Superprecipitation was determined as optical density at 620 nm of muscle actomyosin in a Gilford recording spectrophotometer. At zero time, optical density was obtained for 0.5 mg of actomyosin in 0.15 M KCl-20 mM Tris-acetate (pH 8.3), in a final volume of 1 ml. Following the addition of 0.1 ml of a solution containing 1 mM ATP and 2 mM MgCl_2, decrease in optical density indicated clearing while increased optical density was caused by superprecipitation (15).

Immunization of Rabbits—Unselected New Zealand strain male rabbits, 2 to 3 kg weight, were immunized over a period of 6 weeks by weekly intravenous injections of 1 mg of Lytron particle coated with 0.2 mg of either heart or muscle myosin prepared from dogs. The total volume of each injection was 1 ml. Groups of two animals each were immunized and bled 8 days after the last injection. Individual sera were tested for antibody content and stored frozen at -20°. Sera were not pooled.
Double Gel Diffusion—Double gel diffusion (18) was performed in 0.85% agarose. To overcome the low solubility characteristics of actomyosin and myosin in physiological buffers, agarose was dissolved either in 0.6 M KCl in 0.05 M Tris-HCl buffer (pH 7.2), or in 0.1 M KCl in 0.04 M sodium pyrophosphate buffer (pH 7.8). These proteins were allowed to diffuse in agarose at room temperature for 72 hours. In both instances precipitin lines were obtained with specific antigens and antibodies. However, improvement in both sharpness of precipitin lines and ease of removal of unbound proteins during washing was found when pyrophosphate was present (see Fig. 8 legend). Dried agarose slides were stained with 0.5% Amido black and destained in 7% acetic acid.

Analysis of Association-Dissociation Data—These data were plotted by Langmuir adsorption isotherm, using \( 1/r \) on 1/\( a \); \( r = \mu \text{ contractile protein adsorbed per mg per Lytron particle, and} \) \( \alpha = \mu \text{ free contractile protein per mg Lytron particle remaining}\) in solution after equilibrium was achieved. Contractile protein concentration was computed as total protein minus contaminating proteins in the preparation. F-actin binding by Lytron particle-bound myosin was independent of Lytron particle concentration and was calculated on the basis of micromolar concentrations of interacting proteins. The molecular weights used for computation were as follows: actomyosin 670,000; myosin 500,000; actin monomer 47,000 (19, 20). All experiments were done in duplicate or triplicate. The best fitting line by least squares was obtained for a series of exponential corrections (\( \alpha \)) of 1/\( a \) to determine which protein provided the highest coefficient of correlation (21). Slopes, intercepts, and their standard errors were derived from least squares statistics. \( N \), the maximum amount of protein that could be adsorbed, was estimated as the reciprocal of the \( y \) intercept. \( K \), the average affinity binding constant at equilibrium, was estimated from the \( y \) intercept divided by the slope value.

RESULTS

Adsorption of Actomyosin—Fig. 2a illustrates the kinetics of adsorption of muscle and platelet actomyosin by Lytron particles. All points of these curves are in the region of protein excess over Lytron particle binding capacity, and more protein was bound from 1.2 mg in solution when the concentration of applied Lytron particles was increased from 1 to 12 mg. The fractional binding of muscle and platelet actomyosin are, however, different by a factor of about 20%. Since the muscle actomyosin preparation contained <5% contaminating protein while the platelet preparation contained 20%, the data are consistent with the assumption that only actomyosin (and not contaminating protein) was bound by Lytron particles. Indeed, computation on this basis indicated that each particle adsorbed from 630 to 1225 muscle actomyosin molecules, and from 550 to 1070 platelet actomyosin molecules (Table I).

The Langmuir adsorption isotherm plots shown in Fig. 2B were constructed after contaminating protein concentrations had been subtracted from total values. Maximal linearity was achieved with an exponential correction for 1/\( \alpha \) (heterogeneity index, \( \alpha \)) of 0.75. The affinity constants at equilibrium for 1 mg of Lytron particles were calculated as 14.5 (±0.1) × 10^6 liters mol⁻¹ and 11.4 (±0.6) × 10^6 liters mol⁻¹ for muscle and platelet actomyosin, respectively (Table I). Maximum protein bound by 1 mg of Lytron particles was 2.43 (±0.05) mol⁻¹ for muscle and 2.38 (±0.08) mol⁻¹ for platelet actomyosin (Table I).

Ca²⁺ATPase activity of both proteins, and Mg²⁺ATPase activity of platelet actomyosin, were preserved after adsorption. The Mg²⁺ATPase activity of Lytron particle bound muscle actomyosin showed a decrease of approximately 40% (Table II).

Similarity between muscle and platelet actomyosins in respect to \( K \) value of Lytron particle binding, maximal number of bound molecules per particle, and Ca²⁺ATPase activity are consistent with the assumption that contaminating proteins in solutions applied to Lytron particles either bound very poorly or did not bind at all.

Dissociation of Actin from Actomyosin Bound by Lytron Particles—When Lytron particles coated with either muscle or platelet actomyosin were suspended in a medium containing 0.2 M KCl and 0.2 mmase dase (pH 6.8) at room temperature for 60 min, actin molecules dissociated and were found in solution. Mg²⁺ATPase activity of the remaining Lytron particle-adsorbed protein was reduced to levels similar to that of myosin in solution, while Ca²⁺ATPase was unaffected (Table III). The actin protein released into solution possessed no ATPase activity but,
**Table I**

Adsorption by Lytron particles (LP) of contractile proteins from rabbit back muscle and human platelets

Kinetic values were calculated from Langmuir adsorption isotherm plots shown in Figs. 2B, 3B, 6B, and 6C. Correlation coefficients exceeded 0.97. LM⁻¹, liters mol⁻¹.

<table>
<thead>
<tr>
<th>Protein Adsorbed</th>
<th>Binding constant at equilibrium K (x 10⁶ LM⁻¹)</th>
<th>Heterogeneity index (α)</th>
<th>Maximum protein to be bound by LP M⁻⁸/mg LP</th>
<th>Molecules per particle</th>
<th>Experimental range of molecules/particle at different levels of saturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle actomyosin</td>
<td>14.5 ± 1.0</td>
<td>0.75</td>
<td>2.43 ± 0.05</td>
<td>1,230</td>
<td>1,225 - 630</td>
</tr>
<tr>
<td>Platelet actomyosin</td>
<td>11.4 ± 0.6</td>
<td>0.75</td>
<td>2.38 ± 0.08</td>
<td>1,200</td>
<td>1,070 - 550</td>
</tr>
<tr>
<td>Muscle myosin</td>
<td>34.7 ± 1.7</td>
<td>0.90</td>
<td>2.37 ± 0.07</td>
<td>1,200</td>
<td>1,180 - 680</td>
</tr>
<tr>
<td>Platelet myosin</td>
<td>29.7 ± 2.9</td>
<td>0.90</td>
<td>2.56 ± 0.03</td>
<td>1,325</td>
<td>1,330 - 630</td>
</tr>
<tr>
<td>Muscle G-actin</td>
<td>6.0 ± 0.6</td>
<td>1.00</td>
<td>0.125 ± 0.008</td>
<td>6,500</td>
<td>6,475 - 2,850</td>
</tr>
<tr>
<td>Muscle F-actin</td>
<td>0.65 ± 0.06</td>
<td>0.70</td>
<td>0.696 ± 0.020</td>
<td>35,500</td>
<td>35,200 - 6,500</td>
</tr>
</tbody>
</table>

**Table II**

ATPase activity of contractile proteins in solution and bound by Lytron particles

Mg²⁺ and Ca²⁺ATPase activity was determined on contractile proteins in solution (free) and after adsorption by Lytron particles (bound). All proteins were treated in identical fashion except for the enzymatic incubation which was 15 min for muscle proteins and 30 min for platelet proteins. The values shown are from duplicate samples of three different preparations of each protein.

<table>
<thead>
<tr>
<th>Protein studied</th>
<th>Free ATPase activity (umole P_i/mg protein)</th>
<th>Ca²⁺</th>
<th>Bound ATPase activity (umole P_i/mg protein)</th>
<th>Ca²⁺</th>
<th>Bound ATPase activity (umole P_i/mg protein)</th>
<th>Mg²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle actomyosin</td>
<td>4.18 ± 0.47</td>
<td>4.76 ± 0.77</td>
<td>4.52 ± 0.89</td>
<td>2.51 ± 0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle myosin</td>
<td>5.61 ± 0.26</td>
<td>4.89 ± 0.57</td>
<td>0.19 ± 0.10</td>
<td>0.17 ± 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet actomyosin</td>
<td>1.03 ± 0.18</td>
<td>1.01 ± 0.10</td>
<td>0.79 ± 0.10</td>
<td>0.72 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet myosin</td>
<td>0.98 ± 0.08</td>
<td>0.97 ± 0.13</td>
<td>0.16 ± 0.04</td>
<td>0.20 ± 0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table III**

ATPase activity of Lytron particle-bound actomyosin before and after dissociation of actin

LP, Lytron particles.

<table>
<thead>
<tr>
<th>Protein adsorbed by LP</th>
<th>ATPase activity (umole P_i/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca²⁺ Intact</td>
</tr>
<tr>
<td>Muscle actomyosin</td>
<td>4.8 ± 0.8</td>
</tr>
<tr>
<td>Platelet actomyosin</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

when returned to these Lytron particles (i.e. myosin-coated), reactivated Mg²⁺ATPase levels to those of actomyosin in solution (Table IV). These data also reinforce the assumption that contaminating protein in actomyosin preparations displayed little tendency to bind to Lytron particles.

*Adsorption of Muscle and Platelet Myosin*—Fig. 3.A shows the kinetics of adsorption of muscle and platelet myosin. The patterns are similar to those for actomyosin (Fig. 2A). The number of muscle and platelet myosin molecules adsorbed per particle ranged from 680 to 1180 and 630 to 1330, respectively.

The Langmuir adsorption isotherm plots (Fig. 3B) were made after subtracting known contaminant concentrations from total protein values. The binding constants for muscle and platelet myosins by 1 mg of Lytron particles were 34.7 (± 1.7) x 10⁶ liters mol⁻¹ and 29.7 (± 2.9) x 10⁶ liters mol⁻¹, respectively. The value for maximal muscle myosin bound by 1 mg of Lytron particles was 2.37 (± 0.07) M⁻⁸ while for platelet myosin this value was 2.56 (± 0.03) M⁻⁸. One particle could bind either 1200 muscle or 1325 platelet myosin molecules. The heterogeneity index for the binding of both proteins by Lytron particles was 0.9.

The Ca²⁺ATPase activity of muscle and platelet myosin ad-
TABLE IV

ATPase activity of Lytron particle-bound contractile proteins after addition of activator protein

Proteins were adsorbed as indicated in text. Lytron particle pellets were resuspended in buffer or a solution containing activator. The ATPase activities shown are from duplicate samples of three different protein preparations.

<table>
<thead>
<tr>
<th>Adsorbed protein</th>
<th>Activator protein</th>
<th>ATPase activity (umole Pi/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ca(^{2+})</td>
</tr>
<tr>
<td>Muscle myosin</td>
<td>Muscle F-actin</td>
<td>4.89 ± 0.57</td>
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<td>Muscle F-actin</td>
<td>Platelet myosin</td>
<td>0.94 ± 0.09</td>
</tr>
</tbody>
</table>

Fig. 3. Adsorption of muscle and platelet myosin by Lytron particles. Muscle and platelet myosin at a concentration of 1.2 mg of total protein per ml were adsorbed by increasing amounts of Lytron particles. Experimental conditions are the same as those described in Fig. 2. A, plot of adsorbed contractile protein by increasing amounts of Lytron particles; B, reciprocal plot of data shown in A. Maximal linearity was achieved for both contractile proteins with \( \alpha = 0.9 \). For muscle and platelet myosin, respectively, intercepts were 0.122 ± 0.006 and 0.132 ± 0.011 while slopes were 4.23 ± 0.070 and 3.92 ± 0.18.

Fig. 4. Sequential adsorptions of muscle myosin by Lytron particles (LP). Muscle myosin (1.1 mg per ml) was adsorbed by 12 mg per ml of Lytron particles. Proteins were adsorbed from 0.05 M Tris-HCl (pH 7.2), 0.37 M KCl buffer. After centrifugation, the pellet of Lytron particles was washed with a buffer containing 0.6 M KCl-0.05 M Tris-HCl (pH 7.2) and resuspended in a fresh solution of muscle myosin at the same concentration. This procedure was repeated four times. Protein concentration was determined at each step before and after adsorption and the amount of protein adsorbed was calculated by difference.

Binding of F-actin to Myosin-coated Lytron Particles—When Lytron particles coated with muscle myosin were mixed with muscle F-actin solubilized in 0.1 M KCl, increasing amounts of F-actin were bound at increasing molecular ratios of actin to myosin. The experimental data are given in Fig. 5A. The data, plotted as a Langmuir adsorption isotherm, are shown in Fig. 5B. The heterogeneity index was 0.75. The affinity binding constant was calculated as 2.78 ± 0.43 \times 10^6 \text{ liters mol}^{-1}. The number of actin monomers in each strand of F-actin bound to 1 molecule of myosin ranged from five to ten. However, maximal activation of Mg\(^{2+}\)ATPase was obtained with 2.1 molecules of actin monomers per molecule of muscle myosin. Lytron particles coated with platelet myosin also showed maximal Mg\(^{2+}\)ATPase activation with muscle F-actin at the same molecular ratio (Table V).

Stability of Myosin-coated Lytron Particles—The Ca\(^{2+}\)ATPase activity of myosin-coated Lytron particles decreased about 50% after storage for 1 week at 4°C and 15 to 25% when preserved in 50% (v/v) glycerol at -20°C.

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Fig. 5. Binding of F-actin by Lytron particle-bound muscle myosin. Identical amounts of myosin adsorbed by Lytron particles were mixed with increasing concentrations of F-actin in a solution containing 0.6 M KCl-0.05 M Tris-HCl (pH 7.2). Bound protein was determined before and after adsorption (---). Mg\textsuperscript{2+} ATPase activity (-----) was determined at each experimental point, as described in text. Maximal ATPase activation was obtained at a bound molecular ratio of actin to myosin of approximately 2. A, plot of binding of actin to myosin and the respective Mg\textsuperscript{2+}ATPase activation at different molecular ratio of myosin and actin; B, reciprocal plot of data shown in A. Maximal linearity was achieved with $\alpha = 0.75$. The intercept was 9.4 ± 1.39 and the slope was 3.38 ± 0.145. The binding constant at equilibrium was 2.78 (±0.429) × 10\textsuperscript{6} liters mol\textsuperscript{-1}.

### TABLE V

<table>
<thead>
<tr>
<th>First protein adsorbed by LP</th>
<th>Second protein adsorbed by LP</th>
<th>Ratio of actin/myosin molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle myosin</td>
<td>Muscle F-actin</td>
<td>2.13</td>
</tr>
<tr>
<td>Platelet myosin</td>
<td>Muscle F-actin</td>
<td>2.13</td>
</tr>
<tr>
<td>Muscle F-actin</td>
<td>Muscle myosin</td>
<td>8.20</td>
</tr>
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</table>

**Adsorption of Muscle G-actin**—Fig. 6A illustrates the adsorption curve obtained using increasing concentration of Lytron particles with fixed amounts of either G-actin or F-actin. Contrary to the adsorption kinetics of myosin, only partial adsorption of G-actin to Lytron particles was observed. The maximal coating of Lytron was 1.26 nmol per mg of Lytron particles, or 6494 molecules per particle. However, even when the density of the coat was 2850 molecules per particle, only 25% of available G-actin in solution had been adsorbed. The Langmuir plot shows a homogeneous straight line ($\alpha = 1$). The affinity binding constant was 6.0 (±0.6) × 10\textsuperscript{6} liters mol\textsuperscript{-1}.

From the reported molecular dimensions of G-actin (20), 55 A × 55 A, 6494 molecules are needed to form a monolayer on one Lytron particle. This value is identical with the calculated experimental value.

As expected, preparations of G-actin used for these adsorption experiments did not possess ATPase activity. Furthermore, addition of G-actin-coated Lytron particles to either muscle or platelet myosin in solution, or to muscle or platelet myosin adsorbed by Lytron particles, did not activate Mg\textsuperscript{2+}ATPase. In contrast, G-actin-coated Lytron particles adsorbed F-actin and formed a system that was indistinguishable from F-actin coated Lytron particles.

**Adsorption of F-actin**—Kinetics of the adsorption of F-actin by Lytron particles (Fig. 6C) reflected the heterogeneity known to exist in F-actin preparations in regard to the number of monomers forming F-actin strands. Accordingly, the heterogeneity index was low, $\alpha = 0.7$. The constant of binding was 0.65 (±0.06) × 10\textsuperscript{6} liters mol\textsuperscript{-1} while the maximal amount of protein which could be bound by 1 mg of Lytron particles was 6.96 (±0.20) × 10\textsuperscript{6} (Table I), corresponding to 35,000 monomers of G-actin per particle (Table I). In the presence of ATP, the residual coat was consistently about 6500 molecules per particle (Fig. 7) and, with 12 mg of Lytron particles, all subunits of the same strand appeared to have been firmly bound by Lytron particles.

**Binding of Myosin to F-actin-coated Lytron Particles**—Lytron particles coated with F-actin could bind either muscle or platelet myosin, and the Mg\textsuperscript{2+}ATPase activity of these preparations (Table IV) was equivalent to that of actomyosin in solution (Table II). At maximal Lytron particle coating by muscle F-actin, 1 molecule of muscle myosin was bound per 8.2 actin subunits (Table V). Lytron particles coated with G-actin could bind additional F-actin from a solution containing 0.1 M KCl and 0.1 mM Mg\textsubscript{Cl\textprime}. Such Lytron particles could then activate the Mg\textsuperscript{2+} ATPase of either muscle or platelet myosin in solution. No activation of Mg\textsuperscript{2+}ATPase was observed in mixtures of Lytron particle-coated myosin and Lytron particle-coated F-actin, but cross-activation was observed between muscle F-actin-coated Lytron particles and platelet myosin in solution, and between
FIG. 6. Adsorption of G-actin and F-actin by Lytron particles. Muscle actins in the monomeric G form and polymeric F form, were adsorbed by increasing amounts of Lytron particles. Actin concentration was 1.0 mg per ml. G-actin was adsorbed from a medium containing 0.2 mM ATP-0.2 mM ascorbate (pH 6.8). F-actin was prepared by polymerization of G-actin after addition of 0.1 M KCl and 0.1 mM MgCl₂. A, plot of adsorbed protein by increasing amounts of Lytron particles (one of two experiments). B, reciprocal plot of all G-actin data. These data were linear without correction (α = 1.0). The intercept was 7.01 ± 0.55. The slope was 1.32 ± 0.10. C, reciprocal plot of F-actin data. Maximal linearity was achieved with α = 0.7. The intercept was 0.316 ± 0.028. The slope was 0.48 ± 0.003.

FIG. 7. Depolymerization of Lytron particle-bound F-actin. Dissociation of actin molecules by depolymerization of Lytron particle-bound F-actin was obtained by stirring Lytron particles (LP) from 60 min at 4°C in a medium containing 0.2 mM ATP and 0.2 mM ascorbate (pH 6.8); 6500 molecules of monomeric G-actin were bound regardless the amount of F-actin adsorbed before depolymerization.

FIG. 8. Double gel immunodiffusion on agarose plates; antibodies to myosin of dog heart and back muscle were obtained after six weekly intravenous injections of 0.2 mg of heart or back muscle myosin adsorbed by 1 mg of Lytron particles in 1 ml of total volume. A, agarose was prepared in a buffer containing 0.15 M KCl-0.05 M Tris-HCl (pH 7.2). A, antiserum to back muscle myosin; B, antiserum to heart muscle myosin; 1, 4, back muscle actomyosin; 2, 5, back muscle myosin; 3, 6, heart actomyosin. B, agarose gel was prepared in a buffer containing 0.04 M sodium pyrophosphate and 0.15 M KCl (pH 7.8) to prevent precipitation of protein observed in a. A, antiserum to dog heart myosin; 1, dog heart myosin; 2, dog muscle actin; 3, dog back muscle myosin; 4, dog heart actomyosin.

Immunochemical Studies of Dog Myosin—Myosin adsorbed by Lytron particles was strongly immunogenic. Lytron particle-carrying bound myosin, prepared from either dog heart or dog back muscle, produced immune responses after six injections, each of 1 mg of Lytron particles coated with approximately 0.2 mg of protein. The antiserum produced a single line of precipitation against homologous antigen on double gel diffusion (Fig. 8, A and B), but specificity was quite restricted. Antibodies to dog heart muscle myosin did not react with myosin from either dog back muscle or human platelets. Antibodies to dog back muscle were equally specific, reacting only with homologous antigen and not with myosin from dog heart.

The antibodies were apparently directed against an antigenic platelet myosin-coated Lytron particles and muscle F-actin in solution.
determinant located at or near the myosin-actin combining site. They did not react with either homologous or heterologous actomyosins but, after dissociation of homologous actomyosin, they reacted with antigenic determinants of the myosin exposed after the dissociation (Table VI).

These antibodies were reactive in superprecipitation of dog back muscle actomyosin. After a clearing phase of approximately 10 min (Fig. 9), the presence of specific antiserum produced concentration-dependent inhibition of superprecipitation (Table VI). The lag period of about 10 min for the clearing phase was necessary for maximal inhibition by antibody. In less than 10 min, lesser inhibition was observed with the same antiserum concentration.

Antiserum inhibited the Ca2+-ATPase of myosin and, if preincubated with myosin, it inhibited actin-activated Mg2+-ATPase of actomyosin (Table VI). When antiserum was incubated with either actomyosin, or actomyosin reconstituted from actin and myosin, the Ca2+ and Mg2+-ATPase activities of these preparations were unaffected (Table VI).

**DISCUSSION**

In the last decade a considerable amount of information has accumulated in regard to the structure of fibrillar contractile proteins (22). Irrespective of source, the actomyosin molecule is a complex of myosin and actin (23, 24). Myosin, which forms the thick filaments of myofibrils, is composed of an axial core of two heavy polypeptide chains (MW = 220,000) that terminate in a globular region containing approximately two or three light chains (25). The globular head of muscle myosin appears to contain asymmetrical halves of subfragment 1 (26), each half containing one light chain and a portion of one heavy chain (27), but no agreement exists about the total light chain composition of platelet or brain myosin (28-30). Activation of myosin Mg2+-ATPase requires F-actin, G-actin being inert, but the minimal number of F-actin monomers required for this activation has not been determined until the present study. The minimal number of actins forming a strand that can cross-bridge with the head segment of myosins for initiation of the force of contraction is not known.

Adsorption of fibrillar proteins by Lytron particles offers a new approach for the study of contractile proteins from a variety of tissue origins. When a protein has been anchored to a solid surface, its interactions with other proteins in solution can be readily defined. Indeed, such solid-liquid biphasic models should be extremely useful for the study of many protein-protein interactions.

Actomyosin, and its major components, myosin and actin, all interact with Lytron particles, and high constants of binding were calculated. Increase in heterogeneity in respect to binding was observed as known spontaneous aggregability of proteins in solution was decreased. For F-actin, the most polydisperse system, $\alpha$ was 0.7; for actomyosins, $\alpha$ was 0.75; for myosins, $\alpha$ was 0.9; and for G-actin, the most uniform of these proteins, $\alpha$ was 1.0. The validity of the mathematical treatment chosen for analysis of binding data is supported by identical values for heterogeneity ($\alpha$) for analogous fibrillar proteins of muscle and platelet origin, and the maximal number of molecules bound per particle. Furthermore, these latter values were in agreement with the theoretical number of molecules expected to form a monomolecular layer, based on the known dimensions of specific muscle contractile protein molecules (19, 30).

The high affinity of binding of contractile proteins by Lytron particles, analogous to that reported for human $\gamma$G-globulin (1), suggests that their adsorption is facilitated by some unique molecular characteristic. Langmuir adsorption isotherms suggest that these molecules are bound by Lytron particles in a uniform manner, forming a monolayer with a predominant orientation that does not block the active sites on these molecules but allows them to retain both their normal enzymatic activity

**Table VI**

Effect of anti-myosin (Ab) on ATPase activity of Lytron particle-bound contractile proteins

Anti-myosin was added 10 min before initiation of ATPase activity by ATP. * indicates that myosin had anti-myosin preincubated for 10 min prior to addition of F-actin.

<table>
<thead>
<tr>
<th>Protein determined</th>
<th>ATPase activity (umole Pi/mg protein)</th>
<th>Ca2+ with Ab</th>
<th>Ca2+ no Ab</th>
<th>Mg2+ with Ab</th>
<th>Mg2+ no Ab</th>
</tr>
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<td>Native actomyosin</td>
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<td>4.85</td>
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<td>5.61</td>
<td>1.22</td>
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and their typical actin-myosin binding patterns. Furthermore, the stability of their binding by Lytron particles permits study of interaction between different fibrillar proteins under a variety of ionic concentrations. This is advantageous since lowering of ionic concentration results in insolubilization of these proteins when they are in solution.

The position of adsorbed myosin molecules at the Lytron particle surface was probably very uniform, and molecular unfolding, as observed with 7G-globulin (2), seems not to have occurred. From the number of molecules adsorbed per particle, it is apparent that the position of these elongated molecules on Lytron particles resembles that observed and postulated for the thick filament of myosin within myofibrils (31). Irrespective of the concentration of the solution of myosin applied to Lytron particles and the number of applications, both the final number of molecules bound per particle and the specific activity of the bound myosin were the same.

When myosin was adsorbed by Lytron particles, its characteristic Ca2+ATPase activity was not altered (Table II), and its ability to bind actin and activate Mg2+ATPase was unchanged in relation to the level achieved with the same protein in solution. Therefore, it is apparent that the head portion of myosin, which carries the sites to bind actin, was free and accessible to interact. This was further substantiated by antisera produced by Lytron particles coated with myosin; these antibodies failed to react with actomyosin and inhibited binding of actin by myosin in supersupernatant experiments.

Myosin adsorbed Lytron particles readily bound F-actin from solution, allowing the kinetics of this reaction to be studied. A certain degree of heterogeneity due to the different sizes of F-actin strands was expected to influence the binding by Lytron particle-myosin. This heterogeneity was calculated to be 0.7. Comparison of the number of F-actin monomers required to generate Mg2+ATPase activity showed that maximal activation occurred when 2.1 subunits of F-actin were bound per myosin molecule (MW 5 × 106). This result is consistent with the existence of two actin binding sites on the head of myosin molecules. As long as monomers of actin forming an F-actin filament are bound together, each of these monomers can bind to myosin. The finding (Table V) that maximal Mg2+ATPase activation is reached when two monomers of actin are bound per molecule of myosin indicates that adjacent actin monomers can cross-link several myosin molecules providing that the molecular distance permits such binding. These cross-linkages may be between actin binding sites of either the same or different myosin molecules. Both are possible in a firm monolayer of myosin molecules bound by Lytron particles. F-actin bound by myosin at a level of two monomers per myosin prohibited subsequent adsorption by these Lytron particles of additional F-actin, even when the F-actin was applied in excess. The affinity binding constant of 2.78 (± 0.43) × 106 liters mol−1 is in excellent agreement with >2 × 106 liters mol−1 previously reported (32).

A different picture emerged from adsorption of F-actin. Low concentration of protein solution applied to Lytron particles resulted in irreversible adsorption of each monomer of the applied F-actin strands, whereas from high concentration F-actin strands were bound with two or more subunits. At maximum concentrations only one monomeric subunit from a strand was apparently bound (Fig. 7). Interestingly, the number of monomers irreversibly bound by Lytron particles was constant, in each instance 6500, a value identical with the maximal number of F-actin molecules adsorbed by Lytron particles.

A higher degree of heterogeneity was observed in the Lytron particle binding of either platelet actomyosin or muscle actomyosin than with platelet or muscle myosin. This, too, probably derives from the spontaneous aggregation of these proteins in 0.6 m KCl under which conditions adhesions were performed. Both the affinity binding constants and the degree of heterogeneity of these two proteins were similar, indicating comparable configurations (Table I).

The myosin moiety of actomyosin complexes was responsible for attachment of actomyosin by Lytron particles. This was concluded when ATP produced dissociation of the complex and the released protein was found to be actin.

The decrease in Mg2+ATPase activity obtained after adsorption of muscle actomyosin warrants some consideration. It is possible that some type of allosteric restriction occurs when muscle actomyosin is adsorbed by the Lytron particle surface. Such a restriction could prevent ATP from reaching an active site to be hydrolyzed. Nevertheless, ATP in solution was not prevented from dissociating actin from adsorbed actomyosin molecules and, once dissociation had occurred, inhibition ceased: reconstituted actomyosin molecules on Lytron particles exhibited normal Mg2+ATPase activity.

Antibodies incited by myosin did not crossreact with actomyosin, a degree of specificity not reported previously for antibodies against either myosin or actomyosin. This specificity reinforced the assumption that, when myosin is bound by Lytron particles, the myosin head protrudes from the surface so that immune responses are directed toward the polypeptide segment which carries, or is very near to the sites for, ATPase activity and binding of actin. These antibodies inhibited the ATPase activity of Lytron particle-bound myosin but had no effect on Lytron particle-bound actomyosin molecules. These antibodies also inhibited superprecipitation of actomyosin, but only after the actomyosin was dissociated by addition of ATP.

Immune responses were elicited much more readily by Lytron particle-myosin than by muscle myosin in complete adjuvant. The latter antigen, in weekly immunization with 5 to 10 mg of protein over a period of 3 to 6 months, incited antibodies which reacted with both myosin and actomyosin preparations (29). The immune responses to Lytron particle-myosin, however, were obtained within a period of only 6 weeks, using only 0.2 mg of Lytron particle-bound protein at each injection, and specificity was directed against antigenic determinants present at or near the myosin binding site for actin.

Further investigation is required to determine (a) whether as found for 7G-globulin (2), partial Lytron particles surface saturation results in stimulation of antibodies to other antigenic determinants, and (b) the more precise location on the myosin molecule of the antigenic determinant. This could be one of the myosin light chains near the actin binding site (33).

It is not clear why Lytron particles were saturated by G-actin only when G-actin was in very marked excess. The maximum amount of G-actin adsorbed by 12 mg of Lytron particles represented only 25% of the protein applied. It is tempting to speculate that G-actin molecules carrying the troponin-tropomyosin system are preferentially adsorbed, which in turn could explain the uniform positioning of G-actin molecules on Lytron particles to permit subsequent binding of F-actin polymers starting from a G-actin monolayer. This problem is under investigation utilizing the Ca2+ATPase-dependent sensitivity of ATPase activity.

It appears that contractile proteins are present in a wide variety of living cells (13, 34–36). However, purification of these constituents is limited by the availability of starting material. Therefore, use of hybrid complexes with either myosin or actin
bound to Lytron particles provides a new tool for their separation, and has been used successfully for separation of brain actin (37).

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