The Binding of Smooth Muscle Myosin Light Chain Kinase and Phosphatases to Actin and Myosin*

(Received for publication, January 23, 1984)

James R. Sellers and Mary D. Pato§‡

From the Laboratory of Molecular Cardiology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205 and the §Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N0W0

Contractile activity in smooth muscle cells is regulated by phosphorylation-dephosphorylation of the 20,000-Da light chain of myosin. In an attempt to better understand the localization in muscle of the enzymes which catalyze the phosphorylation-dephosphorylation process, we measured the binding constants of turkey gizzard smooth muscle myosin light chain (MLC) kinase and smooth muscle phosphatases (SMP) to myosin and actin under identical conditions by a sedimentation method. We have observed that MLC kinase binds strongly to both actin and myosin. When tropomyosin is complexed to actin, the affinity of MLC kinase to actin increases 2–3-fold. The presence of calcium-calmodulin weakens the binding of MLC kinase to actin, actin-tropomyosin, and myosin by about 3-fold. Increasing the ionic strength of the binding assay also decreases the binding of MLC kinase to myosin and actin-tropomyosin. MLC kinase is observed to bind to rod subfragment, a fragment of myosin which does not contain the phosphorylatable light chain suggesting that the kinase also binds to domains of the myosin other than the 20,000-Da light chain. Of the phosphatases tested, only SMP-III and -IV bind strongly to unphosphorylated myosin. When the myosin is thiophosphorylated, the binding constants of SMP-III and -IV increase dramatically. SMP-I and -II do not bind to unphosphorylated and thiophosphorylated myosin. However, the free catalytic subunit of SMP-I binds weakly to thiophosphorylated myosin. None of the phosphatases binds to actin. Our study suggests that in muscle, the myosin phosphatase is localized in the thick filament while the MLC kinase may be associated with the thick filaments, thin filaments, or even both.

The contractile state of muscle is thought to be controlled by modulating the level of myosin phosphorylation through the action of kinase and phosphatase activities (1, 2). The enzyme which catalyzes this phosphorylation, myosin light chain kinase, is calmodulin-dependent and is specific for the 20,000-Da light chain of myosin. MLC kinase from gizzard smooth muscle has been isolated to apparent homogeneity and is well characterized (3–5). It has a molecular weight of 130,000, binds 1 mol of calmodulin/mol, and is itself a substrate for cAMP-dependent protein kinase. The phosphatase that dephosphorylates myosin has not been unambiguously identified. However, Pato and Adelstein (6–8) have isolated four phosphatases from turkey gizzard smooth muscle which dephosphorylate the isolated myosin light chains. These enzymes were termed smooth muscle phosphatase I, II, III, and IV. SMP-II is a Mg2+-dependent enzyme which does not dephosphorylate intact myosin (8). SMP-I which is composed of three subunits (Mr = 60,000, 55,000, and 38,000) also does not dephosphorylate intact myosin (6, 7). However, under certain conditions, the catalytic subunit (Mr = 38,000) can be dissociated from the two higher molecular weight subunits and becomes active toward intact myosin. Both SMP-III and SMP-IV are capable of rapidly dephosphorylating intact myosin (7).

There are some questions as to the localization of the kinase and phosphatase in the muscle. Antibodies against smooth muscle MLC kinase have been shown by immunofluorescence to bind to the I-band or actin-containing structures in vertebrate skeletal muscle (9, 10) while no binding to the A-band was observed. Similar experiments with smooth muscle were inconclusive due to the lack of well defined sarcomeric structures (10). On the other hand, another study using MLC kinase antibodies demonstrated MLC kinase to co-localize with myosin in the stress fibers of fibroblasts (11). Two studies using an in vitro assay system at approximately physiological ionic strengths have demonstrated an apparently strong binding of smooth muscle MLC kinase to actin (12, 13). In one of these studies, only weak binding of MLC kinase to myosin was observed under the same conditions (12). There is no information on the localization of the phosphatase. Therefore, we decided to determine the binding constants of smooth muscle MLC kinase and phosphatases to myosin and actin under identical conditions in hopes of understanding the possible intracellular localizations of these enzymes.

MATERIALS AND METHODS

Preparation of Proteins—Smooth muscle myosin and heavy meromyosin were prepared as previously described (14). Both were free of contaminating kinase and phosphatase activities. Phosphorylated myosin (0.95 mol of P/mol of 20-kDa light chain) was prepared by incubating the crude myosin (prior to Sepharose 4B chromatography) in 50 mM NaCl, 10 mM MgCl2, 10 mM Mops (pH 7.0), 10 mM MgCl2, 5 mM ATP, 0.3 mM CaCl2, 0.1 mM EGTA, 5 × 10–4 M added calmodulin, 1 mM dithiothreitol for 10 min at 25 °C in a volume of 10 ml at a protein concentration of 10 mg/ml. The mixture was then made 0.5 M in NaCl, clarified by centrifugation at 40,000 × g for 20 min, and then applied to a Sepharose 4B column (5 × 90 cm) equilibrated at a flow rate of 120 ml/h with 0.5 M NaCl, 10 mM Mops
HMM was phosphorylated as previously described with [γ-32P]ATP (14). Myosin rod subfragment was prepared by digesting myosin (20 mg/ml) with papain (7.0 μg/ml) in 50 mM NaCl, 10 mM Mops (pH 7.0), 1 mM MgCl₂, 0.1 mM EGTA, and 1 mM dithiothreitol, for 10 min at 25 °C. The reaction was stopped with the addition of iodoacetate to a final concentration of 5 mM. The reaction mixture was chilled on ice, made 10 mM in MgCl₂, and centrifuged for 20 min at 40,000 × g. The pellet was washed once in 50 mM NaCl, 10 mM Mops (pH 7.0), 1 mM dithiothreitol, 0.1 mM EGTA, resuspended in, and dialyzed overnight against a large volume of 50 mM NaCl, 1 mM MgCl₂, 10 mM Mops (pH 7.0), 1 mM dithiothreitol, 0.1 mM EGTA. The rod fragment was found to be free of both undigested myosin and subfragment 1 when analyzed by 12.5% and 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Total myosin light chains were prepared from myosin by dissociation with 0.1 M NaHCO₃, followed by ethanol precipitation of the heavy chains. The 20,000-Da and 17,000-Da light chains were resolved by chromatography on hydroxylapatite (15). Myosin light chain kinase was prepared according to Adelstein and Klee (4), calmodulin according to Adelstein (16), and smooth muscle tropomyosin as described by Brandt et al. (21). Protein concentrations for some proteins were determined spectro-photometrically at 280 nm using the following absorbances and molecular weights: myosin, ε₂₈₀ = 0.92 cm²/mg, Mₛ = 237,000/site; HMM, ε₂₈₀ = 0.647 cm²/mg, Mₛ = 167,000/site; rod subfragment, ε₂₈₀ = 0.22 cm²/mg, Mₛ = 130,000/molecule; tropomyosin, ε₂₈₀ = 0.22 cm²/mg, Mₛ = 42,000; troponin, ε₂₈₀ = 0.22 cm²/mg, Mₛ = 70,000; bovine serum albumin, ε₂₈₀ = 0.68 cm²/mg. The concentrations of all other proteins were determined by Bio-Rad Protein Reagent using bovine serum albumin as standard. The bovine serum albumin used in this assay as well as in the binding buffers was monomeric (Fraction V) purchased from Miles Laboratories.

Smooth muscle phosphatase I, the free catalytic subunit of SMP-I, SMP-II, -III, and -IV, were prepared from turkey gizzard smooth muscle. SMP-I and -II were purified to apparent homogeneity according to the procedure of Pato and Adelstein (6-8). Homogeneous fractions of SMP-I were prepared from an impure solution of SMP-I by ethanol precipitation as described by Brandt et al. (21). SMP-III and -IV were prepared in the same manner as SMP-I, using a thioephosphorylated myosin light chain affinity column as the final step in the purification (7). SMP-I and SMP-IV were completely separated by chromatography on ω-aminoethyl Sepharose. SMP-II and -IV obtained by these procedures were highly purified but not homogeneous.

Binding Measurements—The binding of MLC kinase to myosin, actin, and actin-tropomyosin in the presence and absence of calcium and calmodulin was measured in 50 mM NaCl, 10 mM Mops (pH 7.0), 1 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EGTA, 0.5 mg/ml of bovine serum albumin at 25 °C in a volume of 100 μl. When calcium and calmodulin were included, their concentrations were 0.2 mM and 0.1 μM, respectively. The tropomyosin-actin complex was prepared by mixing the two proteins in a 16:1 molar ratio. The concentration of actin or myosin was varied, while that of the kinase was kept constant at 40 nM. The proteins were incubated for 5-10 min at 25 °C and then sedimented at full speed (30 psi) in a Beckman airfuge for either 15 min (for myosin) or for 20 min (for actin). Aliquots were taken from the supernatants, placed on ice, and assayed for MLC kinase activity by incubation of activity stirring in the supernatant for 30 min with 50 mM Tris·HCl (pH 7.4), 4 μM Mg(CH₃CO)₂, 0.2 mM CaCl₂, 10⁻⁷ M calmodulin, 25 μM total myosin light chains at 25 °C. This value was compared to controls where the kinase was sedimented in the absence of actin or myosin. The controls showed that MLC kinase did not sediment in the absence of actin or myosin. Furthermore, a plot of micromoles of 32P transferred/min versus micromolar concentrations of kinase was linear over a wide range of kinase concentrations.

The binding of the smooth muscle phosphatases to myosin, thioephosphorylated myosin, and actin were measured under the same conditions used for the determination of binding of MLC kinase to myosin and actin. The concentrations of the phosphatases in the final binding mixtures were: SMP-I, 3.5 μg/ml; catalytic subunit of SMP-I, 10 μg/ml; SMP-II, 9.5 μg/ml; SMP-III, 5 μg/ml, and SMP-IV, 5.5 μg/ml. Following incubation, the supernatant fractions for phosphatase activity toward the phosphorylated myosin or myosin light chains in a mixture containing the substrate and 50 mM Tris·HCl (pH 7.0) at 25 °C. When the binding mixtures were sedimented in the absence of myosin or actin, all the phosphatase activity remained in the supernatant and was stable during all the experimental manipulations. Examination on glycerol gels of the pellet obtained from the binding experiments using thioephosphorylated myosin indicated no significant dephosphorylation of the myosin during the experiment.

Although SMP-III and -IV used were not homogeneous, determination of their binding constants to myosin and actin by the sedimentation techniques is valid since calculation of these values is based only on fractional binding. Any contamination of SMP-III and SMP-IV by SMP-I and -II can be eliminated by assaying the supernatant for activity towards phosphorylated myosin and myosin light chains. SMP-I and -II have activity against phosphorylated myosin (7). The binding constant for each experiment was derived by a computer analysis of the data using the Marquardt compromise (22).

Determination of Kₐ and Vₐₕ₉—These kinetic parameters were measured from Lineweaver-Burk plots using HMM and 20,000-Da light chains as substrates for MLC kinase under similar ionic conditions as those used in the binding experiments (50 mM NaCl, 10 mM Mops (pH 7.0), 1 mM MgCl₂, 0.1 mM EGTA, 0.2 mM CaCl₂, 0.5 mM MgATP, 0.1 μM calmodulin, 1 μM MLC kinase, 1 mM dithiothreitol at 25 °C).

The Kₐ for the dephosphorylation of phosphorylated HMM was measured in the absence of H₂¹⁷₀₃₀ and -IV and -IV, using a thioephosphorylated myosin light chain solution in 50 mM NaCl, 10 mM Mops (pH 7.0), 1 mM MgCl₂, 0.1 mM EGTA, 0.2 mM CaCl₂, 0.5 mM MgATP, 0.1 μM calmodulin, 1 μM MLC kinase, 1 mM dithiothreitol at 25 °C.

RESULTS

Binding of MLC Kinase to Myosin—One method for determining the binding of a soluble protein such as MLC kinase to an insoluble, filamentous protein such as myosin or actin is by sedimentation of actin or myosin in the presence of the kinase. The fractional enzymatic activity remaining in the supernatant after sedimentation is used to determine the amount of MLC kinase bound. The following control experiments serve to validate this technique. 1) Myosin or actin is virtually completely sedimentable by the high speed centrifugation under the experimental conditions used as indicated by the lack of protein in the supernatant after sedimentation of a myosin solution in the absence of bovine serum albumin. 2) The enzymatic activity of the supernatants obtained by the lack of protein in the supernatant after sedimentation of a myosin solution in the absence of bovine serum albumin is demonstrated by the absence of binding constant of the bound and the ordinate intercept is the reciprocal of the fraction bound. Extrapolation of the data to infinite myosin (or actin) concentration shows that all of the MLC kinase is bound (Fig. 1) as indicated by the unity intercept.

Fig. 1 shows that binding of MLC kinase to thioephosphorylated myosin filaments in the absence of calcium is rather strong. The binding constant was determined to be 1.3 × 10⁶ M⁻¹ (Table I). Interestingly, the binding of MLC kinase to myosin in the presence of 10⁻⁷ M calmodulin and 0.2 mM
Binding of MLC Kinase and Phosphatases

**Fig. 1 (left).** Binding of MLC kinase to smooth muscle myosin. Binding conditions in the absence of Ca\(^{2+}\)–calmodulin (Cam) (circles) are 50 mM NaCl, 10 mM Mops (pH 7.0), 1 mM MgCl\(_2\), 1 mM dithiothreitol, 0.1 mM EGTA, 0.5 mg/ml of bovine serum albumin, 40 nM MLC kinase, while those in the presence of Ca\(^{2+}\)–calmodulin (Δ—Δ) are the same as above but with 0.2 mM CaCl\(_2\) and 0.1 μM calmodulin. The data in the absence of Ca\(^{2+}\)–calmodulin (Ο—Ο, ■—■) were obtained from different MLC kinase and myosin preparations. Both experiments were conducted at room temperature which was approximately 25 °C. See “Materials and Methods” for detailed procedures. The measured binding constants are 1.3 \(\times\) 10\(^6\) in the absence of Ca\(^{2+}\)–calmodulin and 4.1 \(\times\) 10\(^5\) in the presence of Ca\(^{2+}\)–calmodulin.

**Fig. 2 (right).** Binding of MLC kinase to smooth muscle myosin rod. Conditions are the same as in Fig. 1 in the absence of Ca\(^{2+}\)–calmodulin. The data (Ο—Ο, ■—■) were obtained from different MLC kinase and rod subfragment preparations. The measured binding constant is 5.2 \(\times\) 10\(^4\) M\(^{-1}\).

### Table I

Summary of binding constants for MLC kinase and phosphatases to various substrates

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Protein</th>
<th>Binding constant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(-\text{Ca}^{2+}/\text{calmodulin})</td>
</tr>
<tr>
<td>MLC kinase</td>
<td>Myosin</td>
<td>1.3 (\times) 10(^6)</td>
</tr>
<tr>
<td></td>
<td>Phosphorylated myosin</td>
<td>(&lt;\text{1})(^0)</td>
</tr>
<tr>
<td>Rod subfragment</td>
<td>5.2 (\times) 10(^4)</td>
<td>(\text{ND}^*)</td>
</tr>
<tr>
<td>Actin</td>
<td>2.5 (\times) 10(^4)</td>
<td>7.0 (\times) 10(^4)</td>
</tr>
<tr>
<td>Actin-tropomyosin</td>
<td>1.0 (\times) 10(^4)</td>
<td>5.2 (\times) 10(^4)</td>
</tr>
<tr>
<td>SMP-III</td>
<td>Myosin</td>
<td>3.8 (\times) 10(^5)</td>
</tr>
<tr>
<td>SMP-III</td>
<td>Thiophosphorylated myosin</td>
<td>1.1 (\times) 10(^4)</td>
</tr>
<tr>
<td>SMP-IV</td>
<td>Myosin</td>
<td>3.6 (\times) 10(^5)</td>
</tr>
<tr>
<td>SMP-IV</td>
<td>Thiophosphorylated myosin</td>
<td>(\text{ND}^*)</td>
</tr>
<tr>
<td>SMP-I</td>
<td>Myosin</td>
<td>(\text{ND}^*)</td>
</tr>
<tr>
<td>SMP-I</td>
<td>Thiophosphorylated myosin</td>
<td>(\text{ND}^*)</td>
</tr>
<tr>
<td>SMP-I, SMP-II</td>
<td>Myosin</td>
<td>(\text{ND}^*)</td>
</tr>
<tr>
<td>SMP-I, SMP-II</td>
<td>Thiophosphorylated myosin</td>
<td>(\text{ND}^*)</td>
</tr>
<tr>
<td>SMP-I, SMP-II</td>
<td>Actin</td>
<td>(\text{ND}^*)</td>
</tr>
</tbody>
</table>

\(^*\) ND, not determined.

\(\text{CaCl}_2\) is weakened about 3-fold (\(K_\theta = 4.1 \times 10^6\) M\(^{-1}\)) (Table I). Since the dissociation constant for MLC kinase and calmodulin is about 1 nM in the presence of calcium, this value represents virtually complete saturation of MLC kinase with calmodulin (4). On the other hand, we could not detect binding of MLC kinase to phosphorylated myosin using this same technique.

![Graph of kinase activity versus light chain concentration](image)

**Fig. 3.** Double reciprocal plots of kinase activity versus HMM or light chain concentration. The conditions are 50 mM NaCl, 10 mM Mops (pH 7.0), 1 mM MgCl\(_2\), 0.5 mg/ml of bovine serum albumin, 0.1 mM EGTA, 0.5 mM MgATP, 0.2 mM CaCl\(_2\), 0.1 μM calmodulin, 1 nM MLC kinase, 1 mM dithiothreitol at 25 °C. The measured \(K_v\) values are 16.3 μM for HMM light chains (Ο—Ο) and 3.4 μM for HMM light chains (Ο—Ο). The \(V_{max}\) is 1.8 μmol/min/mg for both light chains and HMM.

To test whether MLC kinase can interact with other domains of the myosin molecule, its binding to rod subfragment was measured. A binding constant of 5.2 \(\times\) 10\(^4\) M\(^{-1}\) was determined from the double reciprocal plot (Fig. 2) suggesting that MLC kinase can interact with some part of the myosin other than the light chain. Since MLC kinase binds to rod aggregates, one might expect the kinetics for phosphorylation of the light chains of HMM to be different from that of phosphorylation of the isolated 20,000-Da light chains. Fig. 3 shows a double reciprocal plot of velocity versus substrate concentration for the phosphorylation of isolated 20,000-Da light chains.
light chains and the bound light chains of heavy meromyosin measured under the same conditions as the binding experiments but with the inclusion of 0.5 mM MgATP. While the $V_{max}$ for the phosphorylation of these two substrates appears to be identical, the $K_m$ for the HMM is about 5-fold smaller than that for the light chains. We chose to make the comparison using the soluble subfragment HMM instead of myosin because the former is a soluble system where the two heads have been shown to be phosphorylated randomly at equal rates while myosin filaments have more complex phosphorylation kinetics (23).

**Binding of MLC Kinase to Actin**—Having demonstrated that MLC kinase binds to unpHosphorylated myosin, we next sought to determine the binding constant of the kinase to actin under the same conditions. Fig. 4 shows double reciprocal plots of the binding of MLC kinase to actin in the presence and absence of calcium-calmodulin. In agreement with earlier reports (12, 13), we find that MLC kinase binds to rabbit skeletal muscle actin. The binding constant was determined to be $2.5 \times 10^6$ M$^{-1}$ (Table I). Again we find that the presence of calcium-calmodulin results in a 3-fold decrease in the binding constant ($K_m(20) = 7.0 \times 10^4$ M$^{-1}$) (Table I).

Actin is complexed with tropomyosin in vivo. We, therefore, measured the binding of MLC kinase to actin-tropomyosin (prepared by mixing the two proteins in a 6:1 molar ratio). The presence of tropomyosin results in an increase in the MLC kinase binding constant ($K_m = 1.0 \times 10^6$ M$^{-1}$) over that observed with actin alone. Again, the presence of calcium-calmodulin decreases the binding constant ($K_m = 5.2 \times 10^5$ M$^{-1}$) (Fig. 4, Table I). These experiments have been repeated using purified turkey gizzard actin and similar results were obtained.

Inasmuch as MLC kinase binds to both actin and myosin, we wanted to determine whether the presence of actin could inhibit the phosphorylation of myosin light chains by MLC kinase. We measured the phosphorylation of the isolated 20,000-Da light chains in the presence and absence of actin at concentrations ranging from 1.5 to 24 $\mu$M. These experiments showed that the rate of phosphorylation was unaffected by the presence of actin even at concentrations where the majority of the kinase would probably be bound to actin (data not shown).

The binding of MLC kinase to myosin and actin has so far been measured at a relatively low NaCl concentration (50 mM). Therefore, we wished to test this binding at higher ionic strengths. Since a number of investigators have shown that myosin can be dissociated from its filamentous form to a folded monomeric form (termed 10 S myosin) under some conditions, such as 150 mM KCl (24–26), it is necessary to quantitatively measure the amount of myosin sedimentable under our higher ionic strength conditions. We find that in 50 and 100 mM NaCl virtually all of the myosin is sedimentable at a wide variety of protein concentrations. However, in 150 mM NaCl, only at protein concentrations greater than about 10 $\mu$M was most of the myosin sedimented. Therefore, we measured the binding of MLC kinase to myosin and actin-tropomyosin in the same buffer as the previous experiments but with either 100 mM NaCl or 150 mM NaCl present. Only myosin concentrations above 12 $\mu$M were used for the binding experiments at 150 mM NaCl. Table II shows that the binding of MLC kinase to both myosin and actin-tropomyosin is weakened by increasing the ionic strength. However, the effect of increasing ionic strength is greater on the MLC kinase-actin interaction than on the MLC kinase-myosin interaction.

**Binding of Smooth Muscle Phosphatases to Myosin**—Four different phosphatases which are active against smooth muscle myosin light chains have been isolated from turkey gizzard (7, 8). We have attempted to characterize the binding properties of these phosphatases to myosin and actin.

Smooth muscle phosphatase I has been purified to apparent homogeneity and has been characterized. It is a trimeric enzyme with subunits of 60,000, 55,000, and 38,000 Da (6). The lowest molecular weight subunit has been shown to possess the catalytic activity (7). SMP-I is active against isolated light chains but not against intact myosin. However, the catalytic subunit isolated following dissociation of SMP-I by freeze thawing in the presence of $\beta$-mercaptoethanol or by ethanol treatment dephosphorylates both the isolated myosin light chains as well as intact myosin (7).

We have tested the binding of both SMP-I and the isolated catalytic subunit to unphosphorylated myosin and were unable to detect appreciable binding of either. Since unphosphorylated myosin is the end product of a dephosphorylation reaction, it is not necessarily surprising that the enzymes do not bind well. Therefore, we tested their binding against myosin which had been thiophosphorylated. No detectable binding was observed using SMP-I, whereas the catalytic subunit binds weakly with an estimated binding constant of approximately $10^8$ M$^{-1}$ (Table I).

Figs. 5 and 6 show the double reciprocal plots of the binding of two other phosphatases active toward myosin, SMP-III and SMP-IV, to unphosphorylated myosin. The binding constants were determined to be $3.8 \times 10^5$ M$^{-1}$ for SMP-III and $3.6 \times 10^5$ M$^{-1}$ for SMP-IV (Table I). When tested the

![Fig. 4. Binding of MLC kinase to actin and actin-tropomyosin. Conditions are the same as in Fig. 1. Binding to actin in the absence of Ca$^{2+}$-calmodulin (CaM) (O---O), measured binding constants is $2.5 \times 10^6$ M$^{-1}$; binding to actin in the presence of Ca$^{2+}$-calmodulin (C), measured binding constant is $7.0 \times 10^5$ M$^{-1}$; binding to actin-tropomyosin (TM) in the absence of calcium (O---O), measured binding constant is $1.0 \times 10^5$ M$^{-1}$; binding to actin-tropomyosin in the presence of Ca$^{2+}$-calmodulin ( ), measured binding constant is $5.2 \times 10^5$ M$^{-1}$.

Actin, $+ Ca^{2+}$/CaM

Actin, $+ Ca^{2+}$/CaM

Actin, no $Ca^{2+}$/CaM

Actin/TM, $+ Ca^{2+}$/CaM

Actin/TM, no $Ca^{2+}$/CaM

[Actin]$^{-1}$, (mM)$^{-1}$

[Binding of MLC Kinase and Phosphatases]
binding of these phosphatases to thiophosphorylated myosin, a dramatic increase in the extent of binding (Figs. 5 and 6) was observed. The binding was so strong that the phosphatases are greater than 90% bound over the range of the thiophosphorylated myosin concentrations we can employ in this study. The computer-derived binding constants were 1.1 × 10^6 M^-1 and 8.0 × 10^5 M^-1 for SMP-III and SMP-IV, respectively, to thiophosphorylated myosin (Table I). Patow and Adelstein purified to apparent homogeneity another phosphatase termed SMP-II (6, 8). This phosphatase has a single polypeptide chain with a molecular weight of 43,000 Da. It is a Mg^2+-dependent enzyme which dephosphorylates isolated light chains but not intact myosin (8). We have been unable to detect significant binding of SMP-II to either unphosphorylated myosin or to thiophosphorylated myosin.

**Discussion**

In agreement with other reports, we have shown that MLC kinase binds strongly to rabbit skeletal muscle F-actin even in the presence of 0.5 mg/ml of bovine serum albumin suggesting a specific interaction between actin and the kinase. This interaction is affected by a number of factors like the presence or absence of Ca^2+-calmodulin and tropomyosin. The presence of tropomyosin increases the binding constants 2-3-fold while the presence of Ca^2+-calmodulin decreases these values.

However, we find that our crude myosin preparations are always contaminated with MLC kinase which is difficult to remove except by gel filtration chromatography of the myosin at high ionic strength. Consistent with this observation is our finding that MLC kinase binds strongly to smooth muscle myosin filaments (K_a = 1.3 × 10^6 M^-1) in 50 mM NaCl. Since ATP tends to depolymerize unphosphorylated smooth muscle myosin filaments (24-26), we have not been able to measure the binding of kinase to myosin under conditions similar to those that would be found in resting muscle (i.e. presence of ATP and very low concentrations of calcium; ionic strength of about 150 mM). However, we have determined the effect of increasing ionic strength on the binding of MLC kinase to actin-tropomyosin and myosin in the absence of ATP. We find that whereas the binding of MLC kinase to each of these is weakened as the ionic strength is increased, the effect on the MLC kinase-actin interaction is greater than that on the MLC kinase-myosin interaction. Raising the NaCl concentration from 50 to 150 mM decreases the binding constant of MLC kinase for myosin about 20-fold but decreases that of MLC kinase to actin-tropomyosin about 50-fold. Since the intracellular actin concentration is higher than that of myosin, one might assume that the predominant intracellular location of MLC kinase in vertebrate smooth muscle is the thin filaments. Localization of MLC kinase on actin will not necessarily preclude its ability to phosphorylate myosin light chains since our studies demonstrate that even at conditions where most of the kinase is bound to actin, the kinase activity is not inhibited. However, many parameters remain to be considered, such as 1) how does the interaction of actin with other actin-binding proteins such as filamin (27) or caldesmon (28) affect the binding of MLC kinase to actin? 2) Are the myosin filaments which are prepared by dialysis of soluble myosin to low ionic strength in the presence of MgCl_2 a good model for in vivo myosin filaments? 3) Are there other myosin

<table>
<thead>
<tr>
<th>NaCl concentration (mM)</th>
<th>K_a (Actin-tropomyosin)</th>
<th>Myosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1.0 × 10^6</td>
<td>1.3 × 10^6</td>
</tr>
<tr>
<td>100</td>
<td>6.8 × 10^5</td>
<td>2.1 × 10^5</td>
</tr>
<tr>
<td>150</td>
<td>2.0 × 10^4</td>
<td>6.8 × 10^4</td>
</tr>
</tbody>
</table>

**Fig. 5.** Binding of SMP-III to myosin and thiophosphorylated myosin. Myosin (5 mg/ml) was thio-phosphorylated for 15 min at 25°C under the following conditions: 50 mM Tris-HCl (pH 7.4), 4 mM Mg(CH_3COO)_2, 0.3 mM CaCl_2, 2 mM adenosine-5'-O-(3-thiotriphosphate), 0.1 mM calmodulin, 1 mM dithiothreitol, 10 mM MLC kinase. Conditions are the same as Fig. 1 except for the absence of MLC kinase. The binding constants are determined to be 3.8 × 10^6 M^-1 for unphosphorylated myosin (□□□□□□) and 2.1 × 10^5 M^-1 for thiophosphorylated myosin (■■■■■■).

**Fig. 6.** Binding of SMP-IV to myosin and thiophosphorylated myosin. Conditions were the same as in Fig. 5. The binding constants were determined to be 5.6 × 10^5 M^-1 for unphosphorylated myosin (□□□□□□) and 1.0 × 10^5 M^-1 for thiophosphorylated myosin (■■■■■■).
filament-associated proteins in smooth muscle which may affect the binding?

One similarity between the binding of MLC kinase to actin, actin-tropomyosin, and myosin is that the presence of calcium-calmodulin results in a 3-fold increase in the binding constants. Sobue et al. (13) have proposed a "flip-flop" mechanism for the interaction of actin-tropomyosin with a 135,000-Da smooth muscle protein which was identified as MLC kinase. This mechanism proposes that in the absence of calcium, MLC kinase binds tightly to actin-tropomyosin whereas in the presence of calcium, the enzyme binds to calmodulin and not to actin-tropomyosin. The 3-fold decrease in actin-tropomyosin affinity we observe when calcium-calmodulin is present using actin from either skeletal or smooth muscle would not be expected to give rise to such an all or none mechanism as described by Sobue et al. (13).

Two recent reports have demonstrated that proteolytic digestion of either skeletal or smooth muscle MLCK kinase can result in the production of a 34,000-40,000-Da fragment which has calmodulin-dependent kinase activity (29, 30). Additionally, a calmodulin-dependent MLCK kinase which migrates as a doublet of Mr = 37,000 and 39,000 on sodium dodecyl sulfate-polyacrylamide gels has been isolated from Limulus, the horseshoe crab (31). Although the molecular weight of the native gizzard MLCK kinase is 130,000, the above studies suggest that a domain of MLCK kinase of about 40,000 is sufficient for calcium-calmodulin-dependent phosphorylation of myosin light chains. The remaining portion of the molecule might be involved in other processes such as regulation and binding to actin or to domains of the myosin other than the light chain itself. Our study showing that the Ke for phosphorylation of HMM is lower than that for phosphorylation of isolated light chains is consistent with this idea. It is known from studies of other myosins that the NH2-terminal portion of the light chain which contains the phosphorylatable serine residue is located near the junction of the S-1 and S-2 regions (32-34). In this regard, it is interesting that MLC kinase also binds weakly to myosin rod subfragment. Here the binding is probably occurring in the S-2 region since light meromyosin which is situated at a considerable distance from the S-1 region would tend to be "buried" within the filament.

That MLC kinase binds to rod but not to phosphorylated myosin (which obviously contains rod) is difficult to explain. Perhaps the negatively charged phosphoserine residue prevents such an unfavorable binding environment for MLC kinase that the relatively weak association of MLC kinase to the rod portion is overcome.

The nature of the phosphatases that dephosphorylate myosin in smooth muscle has remained elusive. Pato and Adelstein (6-8) have purified and characterized two different phosphatases (SMP-I and SMP-II) that can dephosphorylate isolated 20,000-Da light chains but not intact myosin. Therefore, it is not surprising that neither of these phosphatases appears to interact with myosin. It is of interest that dissociation of the catalytic subunit of SMP-I results in its activation toward intact myosin. This difference in the substrate specificity of the holoenzyme and catalytic subunit is reflected in their binding properties of thio phosphorylated myosin. Whereas the holoenzyme does not bind to myosin, the catalytic subunit exhibits some binding. Two other phosphatases that dephosphorylate myosin are SMP-III and -IV. Both enzymes bind tightly to myosin. The differences in the binding properties of the catalytic subunit of SMP-1, -III, and -IV are also reflected in their Ke for phosphorylated HMM. The Ke of the catalytic subunit of SMP-I is much higher than those of SMP-III and -IV.

The binding constants of SMP-III and SMP-IV for myosin are increased 25-30 times when myosin is thio phosphorylated. Since these enzymes dephosphorylate thio phosphorylated myosin very slowly, this form of myosin represents a good model for the true substrate for the phosphatase. Therefore, as expected, the binding constants for the thio phosphorylated myosin are higher than those for the unphosphorylated myosin. These observations suggest that either SMP-III, SMP-IV, or both and not the catalytic subunit of SMP-I is the enzyme most likely involved in dephosphorylation of myosin in vivo.

In this study, we have demonstrated that all the components of the myosin phosphorylation-dependent regulation of smooth muscle are capable of associating with the thick filament. Both MLCK kinase and myosin phosphatases (SMP-III and -IV) exhibit high binding constants for myosin. Since none of the phosphatases binds to actin, one could conclude that whichever phosphatase is involved in the in vivo process of myosin dephosphorylation is localized in the thick filament. On the other hand, MLCK kinase binds strongly to both myosin and actin. The degree to which MLCK kinase is distributed between actin filaments and myosin filaments is difficult to predict until more information is presented on how the association of other actin binding proteins and myosin binding proteins affect the interaction of MLCK kinase to actin and myosin.

Acknowledgments—We are grateful to Dr. Robert S. Adelstein for his encouragement throughout the course of these experiments. We thank Dr. M. E. Payne for the gift of some of the MLCK kinase preparations used for these experiments. We gratefully acknowledge the excellent technical assistance of Estelle V. Harvey and the expert editorial assistance of Holly R. Plummer.

REFERENCES