Purification and Characterization of a Smooth Muscle Myosin Phosphatase from Turkey Gizzards*

Mary D. Pato and Ewa Kerc

From the Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N OWG

A phosphoprotein phosphatase that dephosphorylates smooth muscle myosin has been purified to apparent homogeneity from turkey gizzards. Smooth muscle phosphatase (SMP) IV has a molecular weight of 150,000 as determined by gel filtration on a Sephadex G-200 column and is composed of two subunits ($M_r$ = 58,000 and 40,000). Although it is active toward a number of proteins, its activities toward the contractile proteins, intact myosin, heavy meromyosin, and isolated myosin light chains are higher than its activities toward phosphorylase $a$, histone H1A, and phosphorylase kinase. SMP-IV preferentially dephosphorylates the $\beta$-subunit of phosphorylase kinase. The properties of the enzyme have been studied using heavy meromyosin, a soluble chymotryptic fragment of myosin, and isolated myosin light chains as substrates. SMP-IV has high affinity for both substrates and is optimally active at neutral pH. Divalent cations, $Ca^{2+}$ and $Mg^{2+}$, activate the dephosphorylation of heavy meromyosin but inhibit the activity toward myosin light chains. Low concentrations of ATP (1-5 mM) activate SMP-IV but concentrations higher than 5 mM are inhibitory. Inhibition of 50% of the activity of the enzyme by NaF and PP$_i$ requires concentrations higher than 10 mM. Rabbit skeletal muscle heat stable inhibitor-2 has no effect on the activity of SMP-IV toward heavy meromyosin, myosin light chains, and phosphorylase $a$.

Phosphorylation-dephosphorylation has been shown to regulate many biological processes (1, 2). One such process is the contractile activity in smooth muscle and nonmuscle cells. An increase in intracellular $Ca^{2+}$ concentration from $10^{-7}$ to $10^{-4}$ M in response to a stimulus results in interaction between actin and myosin and subsequently, in contraction. There is evidence from in vitro work as well as studies using intact muscle and skinned muscle fibers supporting the hypothesis that $Ca^{2+}$-calmodulin-dependent phosphorylation of the 20,000-Da light chains of myosin is a prerequisite for the actin activation of the myosin $Mg^{2+}$-ATPase activity and for contraction (for reviews see Refs. 3-5).

Myosin light chain kinase, the enzyme which catalyzes the phosphorylation of the 20,000-Da light chain of myosin, has been purified from various sources and is well characterized (3). It is specific for myosin and requires $Ca^{2+}$ and calmodulin for activity. Modification of myosin light chain kinase by phosphorylation with cAMP-dependent protein kinase in vitro decreases its affinity for calmodulin and therefore its activity (6). This property may provide a mechanism for hormonal modulation of contractile activity in smooth muscle and nonmuscle cells.

On the other hand, the enzymes which dephosphorylate myosin and myosin light chain kinase are not as well understood. We have reported the presence of 4 different phosphoprotein phosphatases in turkey gizzard extract. Two of these enzymes, smooth muscle phosphatase I and II, have been purified to apparent homogeneity (7-10). SMP-I is composed of 3 subunits ($M_r$ = 60,000, 55,000, and 38,000) while SMP-II is a single subunit enzyme ($M_r$ = 43,000). Although both enzymes have high activity toward the isolated myosin light chains, they do not act on intact myosin. Thus, the role of SMP-I and -II in muscle contraction is not clear. Because it is active against myosin light chain kinase, it has been suggested that SMP-I may be involved in restoring the high affinity of myosin light chain kinase for calmodulin following phosphorylation by cAMP-dependent protein kinase (8). The observation that the free catalytic subunit of SMP-I is active toward intact myosin indicates that it could play a role in relaxation by dephosphorylating myosin in vivo. However, a physiological mechanism for the dissociation of the catalytic subunit of SMP-I has yet to be found.

The two other phosphatases present in turkey gizzard extract are SMP-III and -IV. As isolated, both enzymes have high activity toward intact myosin, suggesting that one of these enzymes or both enzymes may function in vivo by dephosphorylating myosin to elicit relaxation. In this paper, we report the purification and characterization of SMP-IV. SMP-IV is composed of 2 subunits ($M_r$ = 58,000 and 40,000). It dephosphorylates several phosphoproteins, but it has a preference for the contractile proteins, intact myosin, and isolated myosin light chains. Comparison of the properties of SMP-I, -II, and -IV reveals that SMP-IV is distinct from the other two smooth muscle phosphatases.

**MATERIALS AND METHODS**

All experiments were performed at 4 °C unless otherwise indicated. Deionized, distilled water and reagent grade chemicals were used. Sephacryl S-300, DEAE-Sephacel, aminoethyl-Sepharose, CNBr-activated Sepharose, and Sepharose 4B were purchased from Pharmacia Fine Chemicals. The alkyl agarose exploratory kits, agarose-C$_9$, and agarose-C$_{2}$NH$_2$ series, and $\epsilon$-amino-2-ethyl-2-ethoxyethanol were bought from Miles Laboratories. Turkey gizzard myosin light chain kinase was a gift of Dr. R. S. Adelstein (National Institutes of Health). Heat stable inhibitor-2 and type 1 phosphoprotein phosphatase prepared from rabbit skeletal muscle as in Ref. 11 and [3P]-labeled phosphorylase a -tais were purchased from The Medical Research Council of Canada and the Saskatchewan Health and Research Board. A preliminary report of this work was presented at the Biophysical Society Meeting, February, 1984, San Antonio, Texas. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*This work was supported by the Medical Research Council of Canada and the Saskatchewan Health and Research Board. A preliminary report of this work was presented at the Biophysical Society Meeting, February, 1984, San Antonio, Texas. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

---

1. The abbreviations used are: SMP, smooth muscle phosphatase; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis($\beta$-aminoethyl) ether)-$N,N,N',N'$-tetraacetic acid.
were gifts of Dr. R. L. Khandelwal (University of Saskatchewan). Rabbit skeletal muscle phosphorylase kinase was a gift of C. J. Pallen and Dr. J. H. Wang (University of Calgary).

Preparation of Substrates—Smooth muscle myosin was prepared from fresh turkey gizzards according to the procedure of Sellers et al. (12). The myosin and light chain kinase were dissociated by treatment with guanidine hydrochloride and the myosin light chains were isolated as in Ref. 13. Heavy meromyosin, a soluble chymotryptic fragment of myosin, was used instead of myosin for most experiments in this study because myosin is relatively insoluble at low ionic strength due to filament formation. Except for the loss of a segment of the rod-like tail of the myosin molecule and as a consequence its ability to form bipolar filaments, the enzymatic and structural properties of myosin and heavy meromyosin are very similar. Heavy meromyosin (gift of Dr. R. L. Khandelwal, National Institutes of Health) was prepared by chymotryptic digestion of turkey gizzard myosin (12). 32P-labeled myosin, heavy meromyosin, and myosin light chains were prepared by phosphorylation with turkey gizzard myosin light chain kinase (8) while 32P-labeled myosin light chain kinase (6), histone IIA (7), and phosphorylase kinase (14) were prepared by phosphorylation with the catalytic subunit of cAMP-dependent protein kinase as previously described.

Phosphatase Assay—Phosphatase activity was monitored by the release of 32P, from the substrates (8). Unless otherwise stated, the dephosphorylation reaction was initiated by addition of the enzyme to the reaction mixture (total volume = 50 μl) containing the substrate in 50 mM Tris-HCl, pH 7.0, incubated at 30 °C. The reaction was terminated by addition of 100 μl of 17.5% trichloroacetic acid and 100 μl of 6 mg/ml bovine serum albumin. Following centrifugation of the resulting mixture, a 200-μl aliquot of the supernatant was added to 4 ml of the scintillation fluid and counted in a Beckman 1211 Minibeta liquid scintillation counter. All phosphatase assays were carried out under linear conditions.

Determination of the effect of the heat stable inhibitor-2 on the activity of SMP-IV and type 1 phosphoprotein phosphatase was carried out by incubating the phosphatase and inhibitor at 30 °C for 5 min. The reaction was started by addition of the substrate and samples analyzed.

Polyacrylamide Gel Electrophoresis—Gel electrophoresis in the presence of SDS as described by Laemmli (15) was carried out in 12.5% microslab gels (16) containing 0.1% SDS. Gel electrophoresis in the absence of SDS was carried out in 3.5% polyacrylamide tube gels by a modified procedure described by Fairbairn et al. (17). Elution of the protein from the nondenaturing tube gel was performed by cutting the gel into 2-mm slices and incubating each slice overnight in 100 μl of 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 0.02 mg/ml of β-lactoglobulin, 1 mM dithiothreitol (7). The extracts were then assayed for phosphatase activity against myosin light chains and heavy meromyosin.

Chromatography on Alkyl Agaroses—Study of the binding properties of SMP-I and -IV to the alkyl-agaroses was carried out with exploratory kits of two homologous series of hydrocarbon-coated agaroses, agarose-Cn and agarose-CnNHz (n denotes the number of C atoms of the ligand) (18-20). A kit is composed of 6 columns, each column containing 1 ml of a resin (n = 0, 2, 4, 6, 8, or 10) in the series. The columns were washed extensively with 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol (Buffer A) containing 20 mM NaCl. Aliquots (0.2 ml) of a partially purified mixture of SMP-I and -IV were applied on the columns. The columns were washed with 1.8 ml of Buffer A containing 20 mM NaCl and sequenced with 0.6 ml of Buffer A containing 0.5 mM NaCl. (b) Buffer A containing 1.5 mM NaCl, and (c) Buffer A containing 1.5 mM NaCl and 50% glycerol. The eluates at each step were collected separately and assayed for phosphatase activity toward myosin light chains. The absorbance of the eluates at 280 nm were determined.

The preparative scale chromatography of SMP-IV was carried out on a 20 × 30 cm column (1.5 × 7.2 cm) equilibrated with 20 mM KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (Buffer B) at about 40 ml/h. The mixture of SMP-I and -IV obtained from the DEAE-Sepacel column was dialyzed against Buffer B and then was applied on the column. Following sample application, the column was washed with Buffer B until the absorbance of the eluate at 280 nm returned to base-line. Then a gradient (total volume = 100 ml) of 20–500 mM KCl was started. At the end of the gradient, the column was washed with 100 ml of Buffer B containing 500 mM KCl and then a gradient (total volume = 300–2000 mM KCl was applied. The volume of the fractions collected during sample application and wash with Buffer B was 4–6 ml. This was reduced to 2 ml for the rest of the run.

Affinity Chromatography—Myosin-Sepharose was prepared by coupling turkey gizzard myosin to CNBr-activated Sepharose according to the procedure recommended by the manufacturer. Myosin which was dialyzed against 0.1 M NaHCO3, 0.5 M NaCl was added to CNBr-activated Sepharose swollen in and washed with 1 mM HCl. The slurry was mixed gently overnight at 4 °C. The coupling reaction was terminated by filtering the slurry in a sintered glass funnel and washing with 0.1 M NaHCO3, 0.5 M NaCl. Unreacted groups were blocked by incubating the resin with 1 M ethanolamine at room temperature for 2 h with gentle mixing. The resin was washed extensively before use with Buffer B containing 1 M KCl and then with Buffer B. SMP-IV fractions from the α-aminooctyl-agarose column were dialyzed against Buffer B and applied on the myosin-Sepharose column (1.5 × 9.5 cm) at 40 ml/h. The column was then washed with Buffer B until the absorbance of the eluates at 280 nm returned to base-line. The bound protein was eluted with Buffer B containing 1 M KCl. the eluates were assayed for phosphatase activity toward heavy meromyosin and myosin light chains.

The highly purified SMP-IV was further chromatographed on an affinity column of thio phosphorylated myosin light chain-Sepharose prepared as previously described (7).

Molecular Weight Determination—The molecular weight of SMP-IV was determined on a Sephadex G-200 column calibrated with standard proteins, catalase, bovine serum albumin, myosin, and chymotrypsinogen (Pharmacia Fine Chemicals). The sample (0.3 ml) was applied on the Sephadex G-200 column (0.9 × 60 cm) equilibrated with 0.3 M KCl, 20 mM Tris-HCl (pH 7.4), 0.1 M EGTA, 0.1 mM EDTA, 1 mM diethiothreitol at 4 ml/h.

RESULTS

Purification of SMP-IV from Turkey Gizzard Smooth Muscle—The initial steps in the preparation of SMP-IV are the same as those for SMP-I up to the chromatography on DEAE-Sepacel step (8, 10). This procedure involves extraction of ground fresh turkey gizzards with 50 mM Tris-HCl (pH 7.6), 10 mM magnesium acetate, 15 mM diethiothreitol, 1 mg/ml of pepstatin, 1 mg/liter of leupeptin, 10 mg/liter of L-1-tosylamido-2-phenylethyl chloromethyl ketone, 10 mg/liter of soybean trypsin inhibitor, 10 mg/liter of benzylaminylmethyl ester, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM diethiothreitol followed by (NH4)2SO4 fractionation. The 30–60% saturation fraction which contains about 90% of the phosphatase activity toward myosin, heavy meromyosin, and myosin light chains, is chromatographed on Sephacryl S-300. SMP-I, -II, and -III elute as separate peaks while SMP-IV coelutes with SMP-I (8). Further purification but not resolution of SMP-I and -IV is achieved by chromatography on DEAE-Sepacel.

In the purification of SMP-I we have reported a partial separation of SMP-I and -IV on ω-aminohexyl-Sepharose (8). Although this procedure led to the separation of homologous series from the SMP-I fractions free of SMP-IV, it was not considered satisfactory because of the incomplete separation of SMP-I and -IV. Furthermore, a significant part of the SMP-I fractions is highly contaminated with SMP-IV, thus lowering the yield of SMP-I. The observation that SMP-I and -IV could be partially resolved by ω-aminohexyl-Sepharose raised the possibility that a better separation of these enzymes might be achieved by chromatography on a more appropriate resin. In order to select the suitable resin, the binding properties of SMP-I and -IV to 2 homologous series of agarooses-C6 and agarooses-C8 were studied. Fig. 1, A-C and D-F show the elution patterns of SMP-I and -IV from agarooses-C6 and agarooses-C8, respectively. The protein profiles (Fig. 1, A and D) show clearly that at low ionic strength essentially none of the proteins bind to agarooses (n = 0),
However, they all bind to the hydrocarbon-coated agaroses. Binding increases with increasing C atom chain length of the ligand. Buffer A containing 0.5 M NaCl completely elutes the proteins from the resins with ligands of \( n = 2 \) and 4, but higher salt concentrations are required to elute the proteins from the resins with hydrocarbon chains longer than 4 C atoms. Since SMP-I does not dephosphorylate myosin while SMP-IV does, it was possible to assess the difference in the binding properties of these two enzymes to the resins by analysis of the phosphatase activity profiles toward myosin and myosin light chains. Complete binding of the two phosphatases to the agarose-\( C_6 \) was observed when \( n \geq 2 \) and to agarose-\( C_4 \)NH\(_2\) when \( n \geq 4 \). Examination of the profiles of elution of SMP-I and -IV by Buffer A containing 0.5 M NaCl reveals differences in the binding of these enzymes to certain resins. Whereas 0.5 M NaCl elutes completely the myosin phosphatase activity from agarose-\( C_6 \) (Fig. 1B) and agarose-\( C_4 \)NH\(_2\) (Fig. 1E), only about 20% of the activity toward myosin light chains is eluted (Fig. 1, C and F). The remaining myosin light chain phosphatase activity are eluted only after application of Buffer A containing 1.5 M NaCl (data not shown).

On the basis of the above results, agarose-\( C_4 \)NH\(_2\) was chosen for the preparative scale separation of SMP-I and -IV. Fig. 2 shows the elution profile of the chromatography on agarose-\( C_4 \)NH\(_2\) of the mixture of SMP-I and SMP-IV obtained from the DEAE-Sephacel column. As predicted from the exploratory experiments, application of the first gradient (20-500 mM KCl) on the column elutes SMP-IV but not SMP-I. Application of a higher salt gradient eluted SMP-I. Thus, this technique results in complete separation of SMP-I and SMP-IV (Table 1). The column fractions were pooled as indicated in Fig. 2 and purifying further by affinity chromatography. Homogeneous preparation of SMP-I is obtained by application of the SMP-I fraction on to thiophosphorylated myosin light chain-Sepharose (7).

Recent study of the binding properties of the smooth phosphatases to myosin revealed that SMP-IV binds very tightly to myosin (\( K_{bind} \text{diss} = 3.6 \times 10^5 \text{ M}^{-1} \)) while SMP-I does not bind to myosin (21). On the basis of this observation, purification of SMP-IV from contaminating SMP-I and other proteins was attempted by chromatography on myosin-Sepharose. Fig. 3A shows that the phosphatase binds to the affinity column while a large peak of protein with little phosphatase activity elutes at the void volume. Replication of the flow-through fractions on the column results in a complete recovery of the enzyme. SDS-polyacrylamide gel electrophoresis of the phosphatase eluted from the column with 1 M KCl shows three major bands (Mr = 150,000, 58,000, and 40,000) and some minor bands (inset of Fig. 3A).

Further purification of this sample on thiophosphorylated myosin light chain-Sepharose yields an elution pattern (Fig. 3B) similar to that obtained from myosin-Sepharose column. The SDS-polyacrylamide gel of the purified phosphatase showed two major bands (Mr = 58,000 and 40,000) which migrate differently from the subunits of SMP-I (inset of Fig. 3B).

To determine which of these bands is associated with the phosphatase activity, the purified SMP-IV was subjected to polyacrylamide gel electrophoresis under nondenaturing condition. Fig. 4 shows the activity profile obtained following protein extraction of the sliced gel and assay of the extracts for phosphatase activity toward heavy meromyosin and myosin light chains. The peaks of activity toward these substrates are localized in the same position suggesting that both activities are inherent of SMP-IV. SDS-polyacrylamide gel electrophoresis of the fractions of the peak of activity shows only two bands (Mr = 58,000 and 40,000) (inset of Fig. 4).
FIG. 3. Affinity chromatography of SMP-IV on myosin-Sepharose (A) and thiophosphorylated myosin light chain-Sepharose (B). The columns of myosin-Sepharose (1.5 x 9.5 cm) and thiophosphorylated myosin light chain-Sepharose (1.5 x 7.0 cm) were equilibrated with 20 mM KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride at 40 ml/h. Following sample application, the columns were eluted with the buffer containing 1 M KCl. The column fractions were assayed for phosphatase activity toward heavy meromyosin (○). The absorbance of the fractions at 280 nm was determined (—). SDS-polyacrylamide gels of the eluted proteins are shown.

suggesting that SMP-IV is composed of these two subunits.

When the purified enzyme was chromatographed on a column of Sephadex G-200 calibrated with various protein standards, its volume of elution was characteristic of a globular protein with a molecular weight of 150,000 (data not shown).

Table I shows the activity of SMP-IV toward heavy meromyosin and myosin light chains at various stages of the purification procedure. Affinity chromatography on myosin-Sepharose and thiophosphorylated myosin light chain-Sepharose results in significant increase in specific activity of SMP-IV. The yield obtained from 500 g of gizzards is about 50 pg of protein. SMP-IV preparations have been found to be stable for at least 6 months at -20 °C in 50% glycerol, 20 mM KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol.

Properties of SMP-IV—Like most phosphoprotein phosphatases, SMP-IV dephosphorylates a number of proteins. However, its activities toward myosin, heavy meromyosin, and myosin light chains are about 16-50 times higher than those toward phosphorylase a, phosphorylase kinase, and histone IIA (Table II). The activity of SMP-IV against some of these substrates is inhibited by 0.13 M salt concentration
**Smooth Muscle Myosin Phosphatase**

### TABLE I

**Purification of smooth muscle phosphatase IV using heavy meromyosin and myosin light chains as substrates**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Concentration</th>
<th>Myosin light chains</th>
<th>Heavy meromyosin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total activity &amp; Specific activity</td>
<td>Purification</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nmol/min &amp; nmol/min/mg</td>
<td>-fold</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nmol/min &amp; nmol/min/mg</td>
<td>-fold</td>
</tr>
<tr>
<td>Extract</td>
<td>1,900</td>
<td>4.9</td>
<td>7,478</td>
</tr>
<tr>
<td>30-60% (NH₄)₂SO₄</td>
<td>90</td>
<td>44.0</td>
<td>6,590</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>630</td>
<td>0.78</td>
<td>11,774</td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td>286</td>
<td>0.53</td>
<td>10,213</td>
</tr>
<tr>
<td>w-Aminooctyl-Sepharose</td>
<td>SMP-IV peak</td>
<td>63</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>SMP-I peak</td>
<td>92</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Myosin-Sepharose</td>
<td>12</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Thiophosphorylated myosin light chain-Sepharose</td>
<td>0.5</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*Followed by concentration on w-aminohexyl-Sepharose and dialysis against 50% glycerol, 20 mM KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol.

### TABLE II

**Activity of smooth muscle phosphatase IV toward various substrates**

| Conditions: 1 µM substrate; pH 7.0; 30 °C. | Specific activity
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4 mM KCl</td>
</tr>
<tr>
<td>Myosin light chains</td>
<td>1.6</td>
</tr>
<tr>
<td>Heavy meromyosin</td>
<td>1.8</td>
</tr>
<tr>
<td>Myosin</td>
<td>ND*</td>
</tr>
<tr>
<td>Phosphorylase α</td>
<td>0.1</td>
</tr>
<tr>
<td>Phosphorylase kinase</td>
<td>0.02</td>
</tr>
<tr>
<td>Histone IIA</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*ND, not determined.

---

**FIG. 5.** Lineweaver-Burk plots of SMP-IV. The activities of SMP-IV toward heavy meromyosin (○) and myosin light chains (●) were determined at increasing substrate concentrations.

**FIG. 6.** Effect of Ca²⁺ and Mg²⁺ on the activity of SMP-IV. The activities of SMP-IV toward heavy meromyosin (Δ, ▲) and myosin light chains (○, ●) at increasing concentrations of Ca²⁺ (○, ▲) and Mg²⁺ (●, △) were determined. The substrate concentration was 1 µM. The activity of SMP-IV in the absence of divalent cations is taken as 100%.

The double reciprocal plots of reaction velocity and substrate concentration reveal that the $K_m$ values of SMP-IV for heavy meromyosin and myosin light chains are 5.9 and 1.5 µM, respectively, while the $V_{max}$ values are 9.5 and 3.4 µmol/min/mg (Fig. 5). The low $K_m$ of SMP-IV for these substrates is consistent with the high binding constant of SMP-IV for myosin and the observation that SMP-IV binds tightly to myosin-Sepharose and thiophosphorylated myosin light chain-Sepharose.

SMP-IV is optimally active at neutral pH. The dephosphorylation of heavy meromyosin is maximal at pH 7.0-7.5 while that of myosin light chains is at pH 6.5-8.0 (data not shown).

The divalent cations Ca²⁺ and Mg²⁺ have an opposing effect on the activity of SMP-IV toward heavy meromyosin and myosin light chains. These metal ions inhibit the activity of SMP-IV toward myosin light chains but activate the dephosphorylation of heavy meromyosin (Fig. 6).

Cohen and his co-workers (2, 22) have proposed criteria for the classification of phosphoprotein phosphatases. One of their criteria is the effect of rabbit skeletal muscle heat stable inhibitors (23, 24) on the activity of the enzymes. Study of the activity of SMP-IV toward rabbit skeletal muscle phosphorylase α in the presence of various concentrations of heat stable inhibitor-2 revealed that inhibitor-2 has no effect on...
Smooth Muscle Myosin Phosphatase

FIG. 7. Effect of heat stable inhibitor-2 on the activity of SMP-IV and type 1 rabbit skeletal muscle phosphoprotein phosphatase. SMP-IV (●) and type 1 phosphoprotein phosphatase (○) were incubated with increasing concentrations of rabbit skeletal muscle heat stable inhibitor-2 for 5 min at 30 °C. The reaction was initiated by addition of phosphorylase a and terminated after 10 min at 30 °C by the addition of trichloroacetic acid. One inhibitor unit is the concentration of inhibitor-2 required to inhibit 50% of the activity of type 1 phosphoprotein phosphatase.

The same observations were obtained when heavy meromyosin and myosin light chains were used as substrates. Fig. 7 also shows that the concentrations of heat stable inhibitor-2 used in the experiment inhibited the activity of a partially purified preparation of type 1 phosphoprotein phosphatase.

Another property used to differentiate phosphoprotein phosphatases is their ability to dephosphorylate the α- or β-subunit of phosphorylase kinase. When phosphorylase kinase labeled with 1 mol of phosphate per α- and β-subunit was dephosphorylated with SMP-IV, the reaction rate plateaus at about 50% dephosphorylation (Fig. 8A). Examination on SDS-polyacrylamide gel of aliquots of the phosphorylated kinase taken at various reaction times shows no degradation and difference in the staining intensities of the α- and β-subunits of phosphorylase kinase (data not shown). However, the autoradiogram of the SDS-polyacrylamide gel (Fig. 8B) reveals that SMP-IV progressively released with time the radioactive phosphate from the β-subunit but not from the α-subunit. This was confirmed by densitometric scan of the autoradiogram (Fig. 8C). At the end of the reaction time, the α-subunit is only slightly dephosphorylated (~10%) whereas the β-subunit is almost completely dephosphorylated.

Effect of Common Phosphoprotein Phosphatase Inhibitors—Most phosphoprotein phosphatases have been found to be inhibited by low concentrations of sodium fluoride, sodium pyrophosphate, and ATP. Study of the effect of these compounds on the activity of SMP-IV toward heavy meromyosin and myosin light chains revealed differences between SMP-IV and the other smooth muscle phosphatases, SMP-I and -II. Unlike SMP-I, which is almost completely inhibited by 10 mM sodium fluoride (9), concentrations up to 20 mM have little effect on the activity of SMP-IV (Fig. 9). Fig. 9 also shows that in contrast to SMP-I, which is almost completely inhibited by 1 mM sodium pyrophosphate, about 13 mM PPi, is required to achieve 50% inhibition of the activity of SMP-IV toward myosin light chains. For reasons that are not clear, we have repeatedly observed inhibition of the dephosphorylation of heavy meromyosin by low concentrations of sodium pyrophosphate (1–5 mM) and then reversal of inhibition by 5–10 mM sodium pyrophosphate. SMP-I and -II are highly inhibited by 5 mM ATP, 90 and 60% respectively (8, 9). In contrast, the activity of SMP-IV toward heavy meromyosin and myosin light chains is activated by concentrations of ATP below 5 mM (Fig. 10). Higher ATP concentrations inhibit the enzyme activity. About 13 mM ATP is required to inhibit 50% of its activity.

DISCUSSION

The purification procedure for the preparation of SMP-IV involves the use of a number of different chromatographic
Smooth Muscle Myosin Phosphatase

FIG. 9. Effect of sodium pyrophosphate and sodium fluoride on the activity of SMP-IV. The activities of SMP-IV toward heavy meromyosin (Δ, △) and myosin light chains (○, ●) were determined at increasing concentrations of sodium fluoride (△, ○) and sodium pyrophosphate (Δ, ○). Substrate concentration was 1 µM. The activity of SMP-IV toward myosin light chains is shown for comparison (□—□). The activity of SMP-IV in the absence of NaF and PP↓ is taken as 100%.

EFFECT OF ATP ON PHOSPHATASE ACTIVITY

FIG. 10. Effect of ATP on the activity of SMP-IV. The activity of SMP-IV toward heavy meromyosin (PHMM) (Δ—Δ) and myosin light chains (PLC) (○—○) were determined in the presence of increasing ATP concentration. The activity of SMP-IV toward heavy meromyosin is shown for comparison (●—●). The activity of SMP-IV in the absence of ATP is taken as 100%.

columns. The two most important of these are chromatography on ω-aminoctylagarose and affinity chromatography on myosin-Sepharose and thiophosphorylated myosin light chain-Sepharose. As previously reported, SMP-I and -IV exhibit similar chromatographic properties on Sephacryl S-300 and DEAE-Sephael (8). Only partial separation of these enzymes was previously achieved on an ω-aminohexyl-Sepharose. However, study of the binding properties of SMP-I and -IV to various alkyl-agaroses has revealed a difference in the binding of these enzymes to some resin under certain conditions. Both enzymes bind to hexyl-agarose and ω-aminoctylagarose at low ionic strength, but only SMP-IV is eluted from the resins by a buffer containing 0.5 M NaCl. SMP-I binds more tightly and requires a higher salt concentration for elution. Thus, this technique successfully separates SMP-I from SMP-IV. Further purification of SMP-IV is achieved by affinity chromatography of SMP-IV on myosin-Sepharose and thiophosphorylated myosin light chain-Sepharose.

SMP-IV is shown to be distinct from SMP-I and -II, the other smooth muscle phosphatases present in turkey gizzard extract. The major difference between these enzymes is that, as isolated, SMP-IV is active toward myosin whereas SMP-I and -II are not. Due to the similarity in the molecular weights of the subunits of SMP-I (Mr = 60,000, 55,000, and 38,000) and SMP-IV (Mr = 58,000 and 40,000), we considered the possibility that SMP-IV may be structurally related to SMP-I. Moreover, we have previously reported that the dissociated catalytic subunit of SMP-IV is active toward myosin (8) and that eIF-2 phosphatase, another enzyme which dephosphorylates myosin, is composed of 2 subunits which co-migrate with the 60,000- and 38,000-Da subunits of SMP-I (25, 26). However, examination of several different preparations of SMP-I and -IV on SDS-polyacrylamide gels repeatedly showed differences in the migration of their subunits. Furthermore, although some monoclonal antibodies raised against the free catalytic subunit of SMP-I cross-react with SMP-IV, some antibodies do not bind to SMP-IV. Other differences between SMP-I and -IV are expressed in the effect of various compounds on their activity. The concentrations of ATP and pyrophosphate required to inhibit 50% of the activity of SMP-I are 1.0 and 0.3 mM, respectively (8). The same degree of inhibition of the activity of SMP-IV by these compounds requires about 13 mM.

While SMP-I preferentially dephosphorylates the α-subunit of phosphorylase kinase, SMP-IV dephosphorylates the β-subunit. This observation may indicate a role for SMP-IV in glycogen metabolism since phosphorylation of the β-subunit results in activation of phosphorylase kinase. Cohen and his co-workers (2, 22) have proposed a classification of phosphoprotein phosphatases which is based mainly on (a) their ability to dephosphorylate the α- or β-subunit of phosphorylase kinase (b) the effect of rabbit skeletal muscle heat-stable inhibitors on their activity, and (c) their substrate specificity. Type 1 phosphatases dephosphorylate the β-subunit of phosphorylase kinase; they are inhibited by the rabbit skeletal muscle heat-stable inhibitor, and have high activity toward phosphorylase "a. Type 2 phosphatases exhibit properties opposite to those of type 1 phosphatases. SMP-IV cannot be classified as either type 1 or type 2 phosphatase because although it dephosphorylates the β-subunit of phosphorylase kinase, it is not inhibited by the heat-stable inhibitor and has low activity toward phosphorylase "a.

A number of laboratories have reported the preparation of myosin phosphatases from various smooth muscles. Werth et al. (27) have purified a 2-subunit enzyme (Mr = 67,000 and 38,000) from aorta. It is unlikely that this enzyme is the same as SMP-IV because the aortic myosin phosphatase requires Mn↓↓ for activity toward myosin whereas SMP-IV has no metal ion requirement. Furthermore, the aortic myosin phosphatase is completely inhibited by 5 mM ATP, pyrophosphate,
and sodium fluoride. A much higher concentration of these compounds is required to inhibit the activity of SMP-IV. DiSalvo et al. (28) have identified a histone- and polylysine-stimulated phosphatase in aortic smooth muscle. Since the physical properties of this enzyme are not yet known, comparison with other smooth muscle phosphatases is not possible at this stage.

Onishi et al. (29) have purified a myosin light chain phosphatase from chicken gizzards which is composed of 3 subunits (M₀ = 67,000, 54,000, and 34,000). Structurally, myosin light chain phosphatase resembles SMP-I but they differ in their activity toward myosin. Myosin light chain phosphatase is active toward myosin whereas SMP-I is not.

Sobieszek and Barylko (30) have also reported the purification of two myosin light chain phosphatases from chicken gizzards. One enzyme has two subunits (M₀ = 100,000 and 30,000) while the second enzyme shows a single band (M₀ = 40,000) on SDS-polyacrylamide gel. The authors believe that the latter might represent a small subunit or an active proteolytic fragment of the enzyme.

Contractile activity in smooth muscle is regulated primarily by reversible phosphorylation of myosin. Sellers et al. (12) have reconstituted the myosin phosphorylation system with purified proteins and demonstrated that phosphorylated myosin exhibits high actin-activated Mg²⁺-ATPase activity while dephosphorylated and unphosphorylated myosins have low activity. Various studies with intact muscle and skinned muscle fibers have shown a direct correlation between phosphorylation of myosin and formation of tension. Relaxation of the muscle has been observed to be accompanied with dephosphorylation of myosin. However, dephosphorylation of myosin does not always lead to relaxation. Murphy and his co-workers (31, 32) have reported evidence for the maintenance of tension in intact muscle and skinned muscle fiber under conditions where myosin has been dephosphorylated.

We have identified 4 different myosin light chain phosphatases in turkey gizzard extract. It seems most likely that SMP-III and -IV but not SMP-I and -II are involved in the process of relaxation in vivo because the former are active toward myosin whereas the latter are not. Furthermore, SMP-III and -IV bind tightly to myosin (Kₛₒₜₚₑᵣ = 3.8 × 10⁸ and 3.6 × 10⁸ M⁻¹, respectively) and more tightly to thio-phosphorylated myosin while SMP-I and -II do not bind to myosin or actin (21).

Recently, Hoar et al. (33) studied the effect of SMP-IV on the contractile activity of chicken gizzard skinned muscle fibers. When a muscle fiber, which was contracted by treatment with Ca²⁺-calmodulin-independent myosin light chain kinase in the absence of Ca²⁺ was incubated with SMP-IV, the muscle fibers relaxed rapidly. Analysis of the myosin phosphate content of the muscle fiber following incubation with SMP-IV showed dephosphorylation of myosin. Using partially contracted skinned muscle fibers, it was demonstrated that SMP-IV also dephosphorylates myosin in the presence of Ca²⁺. However, this resulted in increased tension formation rather than relaxation. All these observations suggest that the physiological role of SMP-IV might be to dephosphorylate myosin to restore the muscle to the relaxed state at the end of a stimulus. Table 1 shows that there is sufficient SMP-IV activity in crude extract to carry out this function. The total phosphatase activity in the crude extract that could be attributed to SMP-IV is 2.1 μmol/min. Based on yields of purified myosin obtained from 500 g of turkey gizzards, it was calculated that there are about 3 g of myosin (6 μmol) in this tissue. Thus, under the assay conditions used in this study, the amount of SMP-IV present in muscle could dephosphorylate myosin completely in 6 min. Under physiological conditions where the substrate concentration is saturating and enzyme activity is optimal, the dephosphorylation of myosin could be achieved in a much shorter time.

Acknowledgments—We wish to thank Dr. Shaul Shaltiel and Dr. Robert S. Adelstein for many useful discussions and suggestions and Sherrill Harvey for excellent editorial assistance.

REFERENCES

*J. R. Sellers, National Institutes of Health, personal communication.