PURIFICATION AND CHARACTERIZATION OF A SMOOTH MUSCLE
MYOSIN PHOSPHATASE FROM TURKEY GIZZARDS

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Abstract

The contraction and relaxation of smooth muscle depends on the reversible phosphorylation of the 20,000 Da myosin light chains (MLC\textsubscript{20}). The phosphorylation reaction, which is catalyzed by myosin light chain kinase and results in contraction, has been well characterized. In contrast, little is known about the dephosphorylation reaction or the myosin phosphatase that catalyzes this reaction. The main objective of this project was to study the enzyme that catalyzes the dephosphorylation reaction. To this end a myosin phosphatase termed smooth muscle phosphatase (SMP)-III was purified from turkey gizzards.

SMP-III is highly specific for myosin and MLC\textsubscript{20}. The limited proteolysis of SMP-III results in a 1.5- and 2.3-fold increase in the $V_{\text{max}}$ for MLC\textsubscript{20} and HMM (heavy meromyosin), respectively. The increase in activity correlates with the loss of a peptide of approximately 1 kDa from the C terminus.

Purified SMP-III has a molecular weight of 390,000 Da as determined by gel filtration chromatography and a catalytic subunit of 38,000 Da as determined from non-denaturing polyacrylamide gel electrophoresis and Western blot analysis. Two proteins of 130 and 22 kDa co-purify with the SMP-III catalytic subunit and may be regulatory subunits of the phosphatase.

SMP-III has structural similarities with type 1 protein phosphatases as determined by immunocross-reactivity and proteolytic peptide amino acid sequencing. However, as isolated, SMP-III is enzymatically distinct
from either type 1 or type 2 phosphatases. Limited tryptic digestion of SMP-III modulates the phosphatase activity such that it behaves like a typical type 1 phosphatase. The change in enzymatic properties correlates with the digestion of the C terminus of the catalytic subunit.

SMP-III activity is modulated by a variety of reagents including divalent cations, nucleotides, NaF, and KCl.

The myosin binding properties of SMP-III were determined and compared with other smooth muscle myosin phosphatases and recombinant protein phosphatase. SMP-III and SMP-IIIc both bind strongly to myosin with $K_{binding}$ of $1.9 \times 10^6$ M$^{-1}$ and $1.9 \times 10^5$ M$^{-1}$, respectively. These results suggest that a regulatory subunit is not required for the association of the phosphatase with myosin. The effect of various reagents on $K_{binding}$ was determined. GTP caused a 1.4 and 4 fold increase in the $K_{binding}$ of SMP-III and SMP-IIIc, respectively.

The effect of GTP on the activity of SMP-III was examined. GTP inhibited SMP-III activity towards MLC$_{20}$ and HMM with an IC$_{50}$ of 400 μM at pH 7.4 and 150 μM at pH 7.0. The mechanism for the effect of GTP was examined. GTP may inhibit SMP-III activity by associating with the 20,000 Da myosin light chains. A second possibility is that GTP increases the affinity of SMP-III for its substrate thereby decreasing the rate of dissociation and its movement from one substrate molecule to the next.

The effect of protein kinase C (PK-C) phosphorylation on several properties of SMP-III was determined. PK-C phosphorylation has no effect on SMP-III activity, its sensitivity to heat stable inhibitor 2 or its binding of SMP-III to myosin.
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<tr>
<td>BAME</td>
<td>Nα-benzoyl-L-arginine methyl ester</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo 4-chloro 3-indoyl phosphatetoluidine salt</td>
</tr>
<tr>
<td>Bis</td>
<td>N,N′-methylene bis acrylamide</td>
</tr>
<tr>
<td>Ca-CaM</td>
<td>calcium-calmodulin complex</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethylether)N,N,N′,N′-tetraacetic acid</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>HMM</td>
<td>heavy meromyosin</td>
</tr>
<tr>
<td>I-1</td>
<td>heat stable inhibitor-1</td>
</tr>
<tr>
<td>I-2</td>
<td>heat stable inhibitor-2</td>
</tr>
<tr>
<td>LMM</td>
<td>light meromyosin</td>
</tr>
<tr>
<td>MLC_{20}</td>
<td>20,000 Da myosin light chains</td>
</tr>
<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
</tr>
<tr>
<td>MOPS</td>
<td>8-(N-morpholino propanesulphonic acid)</td>
</tr>
<tr>
<td>NBT</td>
<td>p-nitro blue tetrazolium chloride</td>
</tr>
<tr>
<td>PK-C</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PP-1</td>
<td>type-1 protein phosphatase</td>
</tr>
<tr>
<td>PP-1C</td>
<td>catalytic subunit of PP-1</td>
</tr>
<tr>
<td>PP-1C'</td>
<td>proteolytic fragment of PP-1 catalytic subunit</td>
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<tr>
<td>PP-1δ</td>
<td>δ isoform of PP-1 catalytic subunit</td>
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<td>PP-1G</td>
<td>glycogen-bound PP-1</td>
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<tr>
<td>PP-1I</td>
<td>PP-1 inhibitor-2 complex</td>
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<tr>
<td>PP-1M</td>
<td>myosin-bound PP-1</td>
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PP-2A  type-2A protein phosphatase
PP-2B  type-2B protein phosphatase
PP-2C  type-2C protein phosphatase
PTK   protein tyrosine kinase
PTP   protein tyrosine phosphatase
SDS   sodium dodecyl sulphate
SMP-I  smooth muscle phosphatase-I
SMP-II smooth muscle phosphatase-II
SMP-III smooth muscle phosphatase-III
SMP-IIIc free catalytic subunit of SMP-III
SMP-III_M myofibril-bound SMP-III
SMP-IV smooth muscle phosphatase-IV
STI   soybean trypsin inhibitor
TCA   trichloroacetic acid
TEMED N,N,N',N'-tetramethylene diamide
TPCK  N-tosyl-L-phenylalaninechloromethyl ketone
1.0 Introduction

Muscles are organs that produce movement through the process of contraction and are essential for many physiological processes. In vertebrates, muscle can be divided into three types, skeletal, cardiac and smooth, each with a distinct role to play. Skeletal muscles, the dominant form of musculature, are responsible for the voluntary movements of the body. Cardiac muscle is responsible for the movement of blood through the arteries and veins. Smooth muscle functions to provide mechanical support to the hollow organs including the blood vessels, airways, and the digestive tract. The contractile state of the smooth muscles influences the flow of material through these organs.

The varied roles are supported by morphological and functional differences. Skeletal muscles are formed from long, multinucleated cells containing a parallel arrangement of fibers (see figure 1.1). The fibers are made up of functional units or sarcomeres composed of interdigitated thick and thin filaments. The thin filaments are anchored at the Z line. Each of these units can contract individually. The level of force development depends on the coordinated contraction of many sarcomeres. Skeletal muscles are under voluntary control responding to nervous stimuli.

Cardiac muscle cells, although much smaller than skeletal muscle cells, also contain well defined contractile units. The cells are joined at an intercalated disk which provides a strong union between cells. The membranes of individual cells fuse to form a bridge known as a gap
junction which permits the coordination of contraction throughout the heart. Pacemaker cells within the heart ensure contractions occur at regular intervals. The rate of contraction can be modulated by nervous and hormonal stimuli.

Figure 1.1. Organization of contractile proteins in striated muscle. This figure was modified from a diagram in Essential Medical Physiology (1992) edited by L. R. Johnson, from the chapter Striated Muscle (p. 86) by N. Weisbrodt, published by Raven Press, used with permission.

Smooth muscles exist in three broad categories, visceral or single unit smooth muscle, multiunit smooth muscle, and intermediate smooth
muscle. Single unit smooth muscle is the dominant form consisting of large sheets of muscle cells joined with many gap junctions. This permits the coordinated contractions without membrane depolarization. Single unit smooth muscle can undergo two types of contraction, phasic or tonic. Phasic smooth muscles are found in the digestive tract where slow peristaltic-like contractions move food along the alimentary canal. In contrast, airways and most blood vessels demonstrate tonic contractions. The normal function of these organs requires the maintenance of a constant pressure. Multiunit smooth muscle contains fewer gap junctions and coordinated contractions are mediated by neuronal stimulation. This type of muscle functions in the tissues requiring precise movements such as the control of the iris. Intermediate smooth muscle have been described in some airway smooth muscle. This smooth muscle type contains gap junctions and responds to both neuronal and hormonal stimuli. All types of smooth muscle are under involuntary control.

Despite their varied roles, the different muscle types have many properties in common. The interaction of actin and myosin is intergral to the contraction of all muscle types. Myosin and actin are arranged into thick and thin filaments, respectively (illustrated in figure 1.1). In skeletal and cardiac muscle, these filaments are arranged into regular arrays that are visible as striations under the light microscope. Smooth muscle filaments are not arranged in such a dense pattern and thus are difficult to identify under the light microscope.

Huxley and Hanson (1954) and Huxley and Neidergerke (1954) proposed the sliding filament model to describe contraction in skeletal muscle. Subsequent investigation has shown that this model also applies to cardiac and smooth muscle. In this model actin and myosin filaments
slide past one another in an ATP-dependent manner. The signal for contraction in all muscle types is an increase in the free cytosolic calcium concentration; however, the mechanisms that mediate the contractile process differ between the muscle types. In skeletal and cardiac muscle, contraction is primarily regulated by the thin filament proteins troponin and tropomyosin. Under resting conditions the tropomyosin blocks the interaction of actin and myosin. Muscle stimulation results in an increase in the free intracellular calcium concentration. The calcium binds to troponin C resulting in the subsequent displacement of tropomyosin allowing interaction between actin and myosin.

In contrast to the situation in striated muscle, regulation of smooth muscle contraction is mediated by the thick filament (figure 1.2). In this tissue the increase in calcium stimulates the calcium-calmodulin (Ca-CaM)-dependent phosphorylation of the 20,000 Da myosin light chains by myosin light chain kinase (MLCK). Unphosphorylated myosin light chain inhibits the interaction of actin (A) with myosin (M). Phosphorylation reverses this inhibition. Actin then interacts with myosin and activates the Mg\(^{2+}\) ATPase activity of the myosin head which hydrolyses ATP and provides the energy required to drive muscle contraction. Relaxation of smooth muscle requires the decrease of calcium concentration to resting levels and myosin dephosphorylation. This process is catalyzed by a specific myosin phosphatase (PP). When this study was initiated the exact nature of this enzyme was unknown. The goal of this project was to purify and characterize the phosphatase responsible for the dephosphorylation of myosin.
Figure 1.2. The mechanism of contraction in smooth muscle. Myosin light chain kinase (MLCK), myosin phosphatase (MP), calcium calmodulin complex (Ca-CaM), myosin (M), actin (A).
2.0 Review of the literature

2.1 Protein phosphorylation-dephosphorylation

The reversible phosphorylation of proteins has long been recognized as an important mechanism for the control of a wide variety of cellular processes. These include glycogen metabolism, muscle contraction, and cell growth and division. Phosphorylation is catalyzed by protein kinases while the dephosphorylation reaction is catalyzed by protein phosphatases.

2.1.0 Protein kinases

Protein kinases were originally described as non-specific enzymes capable of phosphorylating proteins containing extended tracts of serine residues (reviewed by Walsh and Krebs, 1973). Studies over the past three decades have described a diverse group of highly regulated enzymes able to phosphorylate serine, threonine or tyrosine residues. Phosphorylation of serine and threonine are catalyzed by one family while phosphorylation of tyrosine is catalyzed by a separate family of enzymes (Taylor et al., 1992). Recently, several dual specificity kinases with the ability to phosphorylate both serine/threonine and tyrosine residues have been described (Lindberg et al., 1992; Nishida and Gotoh, 1993).

Common to all kinases is a highly conserved catalytic core, each containing a glycine loop and conserved aspartate and lysine residues (Taylor et al., 1992). Serine/threonine kinases can be divided into several
general groups by virtue of their substrate specificity and regulatory requirements (see review by Kennely and Krebs, 1991). Substrate specificity can be summarized by a sequence of amino acids surrounding the phosphorylation site that are required for substrate recognition and phosphorylation known as a consensus sequence. cAMP-dependent protein kinase, the best studied of the kinases, and cGMP-dependent protein kinase are regulated by cyclic nucleotides. Both cAMP- and cGMP-dependent protein kinases phosphorylate a serine or threonine residue that is located one or two amino acid residues on the C-terminal side of a positively-charged residue. The consensus sequence for cAMP dependent protein kinase has been described as R-R/K-X-S/T > R-X2-S/T = R-X-S/T where X is any amino acid. The consensus sequence for cGMP dependent protein kinase is (R/K)2-3-X-S/T. Protein kinase C is a family of enzymes that require calcium, diacylglycerol, and phosphatidylserine for activity. Substrates of protein kinase C contain the more complex consensus sequence (R/K1-3)2-X2-0)-S/T-(X2-0,R/K-1-3) > S/T-(X2-0,K/K-1-3) ≥ (R/K1-3,X2-0)-S/T. Myosin light chain kinase, calcium-calmodulin dependent protein kinase II, and glycogen phosphorylase kinase all require calcium and calmodulin for activity although they differ markedly in substrate specificity and subunit composition. The smooth muscle myosin light chain kinase is highly specific for the smooth muscle 20 kDa myosin light chains. This specificity is reflected in the complex nature of the consensus sequence (K/R,X)-X1-2-K/R3-X2-3-R-X2-S-N-V-F while the broad substrate specificity of calcium-calmodulin dependent protein kinase II is reflected in the less restrictive consensus sequence R-X-X-S/T. The consensus sequence for glycogen phosphorylase kinase has not been determined. Cell division cycle (cdc) 2 kinase is a proline-directed kinase has a
consensus sequence of S/T-P-X-R/K where X is any polar amino acid reflects the proline requirement.

Most of the protein tyrosine kinases (PTKs) have been identified through the screening of genetic libraries. Two main classes of protein tyrosine kinases have been described, the transmembrane receptor kinases and the Src related PTKs (Hunter and Cooper, 1985). Receptor tyrosine kinases, which include the insulin receptor, epidermal growth factor receptor, and the growth hormone receptor, are involved in the transduction of extracellular signals that regulate cell growth and metabolism. Binding of a ligand to the extracellular domain induces a conformational change resulting in the activation of the intracellular tyrosine kinase activity. Although these kinases are known to autophosphorylate, the exact nature of their substrates remains unclear. The Src PTKs, first identified as the transforming factor in retroviral infections, have their origin in vertebrate genes. The best known examples, the Rous sarcoma virus protein p60\textsuperscript{v-src} and its cellular homolog p60\textsuperscript{c-src}, are soluble proteins that associate with the membrane through an N-terminal myristyl group. Similar to the receptor kinases the Src PTKs autophosphorylate on tyrosine residues.

2.1.1 Protein phosphatases

Protein phosphatases are less well characterized than the protein kinases. Phosphatases catalyze the hydrolysis of the hydroxyl bound phosphate of phosphoproteins. As with protein kinases, protein phosphatases can be divided into two families, enzymes that remove phosphate from serine/threonine (Cohen, 1989) and those that dephosphorylate tyrosine (Fischer et al., 1991). Recently several dual
specificity protein phosphatase have been described (Hannon et al., 1994; Ward et al., 1994).

The protein tyrosine phosphatases (PTPs) have been classified as either membrane bound receptor-like or nontransmembrane proteins. At least one copy of a highly conserved catalytic domain is present in each enzyme (Charbonneau and Tonks, 1992) containing the active-site consensus motif [I/V]HCXAGXXR[S/T]G (Mauro and Dixon, 1994). PTPs have very high specific activities towards a wide range of substrates in vitro (Fischer et al., 1991). This activity necessitates some form of regulation to prevent indiscriminate dephosphorylation in vivo. Unique sequences which have been identified on the PTPs are responsible for directing the enzymes to specific subcellular locations (Mauro and Dixon, 1994). This targeting mechanism allows for the sequestration of the phosphatase when not in use and ensures the dephosphorylation of the appropriate substrate.

The serine/threonine protein phosphatases are the predominant form of phosphatases found in the vertebrate cell and are the main focus of this section.

2.1.1.0 Classification of the serine/threonine protein phosphatases

Protein phosphatases have been the subject of extensive investigation for more than a decade (see Cohen, 1989; Shenolikar and Nairn, 1991). Early investigations focused on the phosphatase as they applied to specific cellular functions. To coordinate the mass of information accumulated on the nature and role of these enzymes a number of classification schemes have been proposed. The most widely used classification scheme was proposed by Ingebritsen and Cohen (1983).
This scheme divides protein phosphatases into two classes based on their enzymological properties. The first criterion used is the ability of the enzymes to preferentially dephosphorylate either the α or β subunit of phosphorylase kinase that has been phosphorylated by cAMP-dependent protein kinase. The second is the sensitivity of the phosphatase to two heat stable inhibitors termed inhibitor-1 and inhibitor-2.

Type-1 protein phosphatases, also abbreviated as PP-1, preferentially dephosphorylate the β subunit of phosphorylase kinase and are sensitive to inhibition by heat stable inhibitors-1 and -2. The free catalytic subunit of PP-1 (PP-1C) has been isolated from liver, skeletal muscle, smooth muscle and ranges from 33,000 to 38,000 Da. The 33,000 Da form is thought to be a proteolytic product of the higher molecular weight form. Several multisubunit complexes have been described for the type-1 phosphatases. The glycogen bound form of type-1 phosphatase (PP-1G) is composed of the 38,000 Da PP-1 catalytic subunit and a 160,000 Da glycogen binding subunit (Stålforß et al., 1985). A myosin associated form (PP-1M) with a 130,000 myosin binding subunit and a 20,000 Da subunit in addition to the catalytic subunit has been identified (Chisholm and Cohen, 1988a; Alessi et al., 1992). Finally, an inactive complex of the type-1 catalytic subunit and the heat stable inhibitor-2 (PP-1I) has been described. PP-1I can be activated through the phosphorylation of the inhibitor-2 subunit. Type-1 phosphatase catalytic subunit has been cloned and sequenced. It has been shown to have ~50% identity to type-2A within the core catalytic region and an overall homology of 59% (Berndt et al., 1987; Bai et al., 1988). Subsequent analysis has shown the existence of at least four type-1 isozymes, α, γ1, γ2, and δ, which have 90% sequence identity (Sasaki et al., 1990).
Type-2 protein phosphatases (PP-2) preferentially dephosphorylate the α subunit of phosphorylase kinase and are not affected by inhibitors-1 and -2. Type-2 phosphatases are further subdivided into types -2A, -2B, and -2C according to their requirement for divalent cations.

Type-2A does not require a metal ion for activity and is further subdivided according to its subunit composition. Type-2A phosphatases can be isolated as a heterotrimer (PP-2A₀ and PP-2A₁) composed of the catalytic or C subunit and two regulatory subunits, designated A and B, or as a dimer of A and C subunits (PP-2A₂) (Cohen, 1989). Types 2A₀ and 2A₁ possess distinct B subunits, 55 kDa B and 54 kDa B’, respectively. Recently, a third B subunit, 74 kDa B‘’, has been identified. The type-2A catalytic subunit can be separated from the regulatory subunits by precipitation with ethanol or by freeze and thaw in 0.2 M mercaptoethanol (Lee et al., 1980). Cross-linking experiments have demonstrated that the B subunit interacts with the AC complex (Kamibayashi et al., 1992) but will not form a complex with the catalytic subunit or A subunit alone. Type-2A phosphatase catalytic subunit was the first to be analysed by cDNA sequencing. Two isoforms with 97% identity, α and β, were isolated from rabbit skeletal muscle (da Cruz e Silva et al., 1987; da Cruz e Silva and Cohen, 1987). Type-2A cDNA has since been isolated from human lung (Hemmings et al., 1988), and liver (Arino et al., 1988), rat liver (Kitagawa et al., 1988), bovine adrenal (Green et al., 1987) and porcine kidney (Stone et al., 1987).

Type-2B is composed of a 60,000 Da catalytic subunit and a 15,000 Da regulatory subunit and has an absolute requirement for Ca²⁺ for activity. This enzyme was first identified by its ability to inhibit cyclic nucleotide phosphodiesterase from brain (Klee et al., 1988). PP-2B bound calmodulin
required for phosphodiesterase activity and, thus, inactivated the enzyme. Subsequently, calmodulin was found to activate PP-2B phosphatase activity in the presence of calcium. The core phosphatase region of type-2B has been cloned from mouse brain and shows 59% identity with both PP-2A and PP-1 (Kincaid et al., 1988). Subsequently two isozymes of type-2B have been identified as α and β which show 91% identity in their phosphatase domains and are 96% identical in their calmodulin binding site (Ito et al., 1989; Kuno et al., 1989; Guerini and Klee, 1989).

Finally, type-2C is a monomer of ~43,000 that requires Mg\textsuperscript{2+} for activity. This enzyme is ubiquitous in vertebrates and is active towards a broad range of substrates. Amino acid sequence analysis and electrophoresis of proteolytic peptides from type-2C suggest the existence of two distinct isoforms (McGowan and Cohen, 1987; McGowan et al., 1987). Subsequent sequence information derived from cDNA clones have shown that these enzymes bear no similarity to types-1, -2A or -2B phosphatases and are, therefore, members of a distinct gene family (Tamura et al., 1989; and Wenk et al., 1992).

Recently, genetic screening techniques have been used to identify distinct protein phosphatases with homology to PP-1, PP-2A, and PP-2B (Cohen et al., 1990; Chen et al., 1992). Significant variations within the homologous central region have been found suggesting that these enzymes possess enzymatic properties distinct from those phosphatases currently being studied.

Several enzymes have been described that do not fit into the Ingebritsen and Cohen classification scheme. A 70 kDa phosphatase has been purified from skeletal muscle with activity towards skeletal myosin light chains (Morgan et al., 1976). This enzyme requires Mg\textsuperscript{2+} or Mn\textsuperscript{2+} for
activity, like PP-2C; however, the enzyme does not dephosphorylate phosphorylase kinase. Wollny et al. (1984) have described a 56 kDa ribosomal S6 phosphatase from rabbit reticulocytes which is dependent on Mn$^{2+}$ for activity. This phosphatase can be inhibited by inhibitor 2 only if the two are first incubated in the absence of Mn$^{2+}$ (Tipper et al., 1986). A similar enzyme from *Xenopus laevis* oocytes has been described by Andres and Maller (1989). This 55 kDa enzyme has ribosomal S6 phosphatase activity in the presence of Mg$^{2+}$ and, like PP-1, is sensitive to inhibitors-1 and -2 but this enzyme is inactive towards phosphorylase kinase. A latent phosphatase, LP-2, has been purified from pig brain that cannot be classified according to the Ingebritsen and Cohen scheme (Yang et al., 1986). LP-2 has a molecular mass of 350,000 Da when determined by gel filtration chromatography but migrates as a single 49,000 molecular weight band on SDS PAGE. The 49,000 Da mass suggests a type-2C enzyme, however, LP-2 does not require either Mn$^{2+}$ or Mg$^{2+}$ for activity. LP-2 is also insensitive to inhibition by inhibitor-2. Honkanen et al. (1991) describe a 36 kDa phosphatase from bovine brain that dephosphorylates the β subunit of phosphorylase kinase but, in contrast to PP-1, is stimulated by inhibitor-2. Amino acid sequence analysis showed some similarity with both PP-2A and PP-1 in one of three peptides derived from digestion with cyanogen bromide. The other two peptides showed no homology to either PP-1 or PP-2A. These examples suggest that the scheme proposed by Ingebritsen and Cohen cannot account for all serine/threonine phosphatases.

An alternate classification scheme has been proposed by Merlevede (1985) based on enzymological properties of the phosphatases. This system employs descriptive names to avoid the confusion created by a numbering
system such as the one utilized by Ingebritsen and Cohen. Four classes have been identified, ATP,Mg$^{2+}$-dependent protein phosphatase, polycation stimulated protein phosphatase, Mg$^{2+}$-dependent protein phosphatase, and calcineurin (Merlevede, 1985). This scheme contains obvious similarities with that of Ingebritsen and Cohen. Calcineurin and the Mg$^{2+}$-dependent protein phosphatase are likely the type-2B and -2C, respectively, while the enzymatic properties of the polycation-stimulated phosphatase suggests PP-2A. ATP,Mg$^{2+}$-dependent phosphatase is reminiscent of the PP-1$_1$ form of type-1 phosphatase.

Based on sequence information, Mumby and Walter (1993) have suggested an alternate classification scheme for serine/threonine protein phosphatases. Sequence similarity of types-1, -2A, and -2B phosphatases define a single genetic family. These enzymes would be referred to as PP-1, PP-2, and PP-3 respectively. Owing to its distinct structural nature, type-2C would be referred to as MP1.

For the sake of simplicity and continuity, phosphatases will be referred to according to the Ingebritsen and Cohen scheme wherever possible.

2.1.1.1 Protein phosphatase inhibitors

Serine/threonine phosphatase activity can be modulated by a variety of inhibitory compounds. The phosphatase inhibitors can be divided into three broad classes depending on their structure. The first class is made up of heat stable proteins of low molecular weight that have been isolated from a variety of vertebrate tissues. A second class of inhibitors are naturally-occurring toxic compounds produced by several aquatic organisms. Although structurally unrelated, these compounds are
all very potent inhibitors of phosphatase 1 and 2A activity. The third class of inhibitors comprise both small organic and inorganic compounds. Phosphatase inhibitors have proven to be a useful tool in elucidating the role of protein phosphatases in many cellular processes. The specificity of some of these inhibitory compounds provides a tool to determine which phosphatase is involved in specific dephosphorylation events.

**2.1.1.1.0 Heat stable inhibitors of PP-1 activity**

Three heat and acid stable polypeptide inhibitors of PP-1 activity have been isolated from mammalian tissue. Two of these, inhibitor-1 and DARPP-32 (dopamine and cAMP regulated phosphoprotein), have many properties in common. Inhibitor-1 (I-1) was first isolated from rabbit skeletal muscle (Huang and Glinsmann, 1976) and has since been purified from rabbit liver (MacDougall et al., 1989) and bovine caudate nucleus (Nairn et al., 1988). Western blot analysis has identified I-1 in a variety of other tissues and animals including the brain, heart, kidney, uterus, and adipose tissue of rabbit and rat (MacDougall et al., 1989). I-1 has also been found in the liver of pig, guinea pig and sheep but is absent from the liver of rats and mice. The inhibitory activity of I-1 is dependent upon the phosphorylation by cAMP-dependent protein kinase at threonine 35 (Huang and Glinsmann, 1976; Aitken et al., 1982). Proteolytic peptide 2-66 retains biological activity indicating the importance of the N-terminus in phosphatase inhibition (Aitken et al., 1982).

DARPP-32 has been isolated from neural tissue (Hemmings et al., 1984). The activity of DARPP-32 is dependent on the phosphorylation of threonine 34 by cAMP-dependent protein kinase (Williams et al., 1986). Antibodies specific for DARPP-32 have shown enrichment in the basal
ganglia and distribution through the neostriatum of the mouse, rat, guinea pig, cow, rabbit, and monkey (Hemmings and Greengard, 1986).

Inhibitor-2 (I-2), first isolated from rabbit muscle (Huang and Glinsmann, 1976), has been identified in rabbit liver, heart, kidney, brain, lung, and diaphragm (Roach et al., 1985). Sequence analysis shows no homology with inhibitor-1 or DARPP-32. I-2 does not require phosphorylation for activity (Holmes et al., 1986). Inhibitor-2 can interact with the PP-1 catalytic subunit forming an inactive complex known as PP-11. This complex can be activated by phosphorylation of I-2 at threonine 72 by glycogen synthase kinase-3 (Holmes et al., 1986).

A property shared by all three heat stable inhibitors is the variance in molecular weights obtained by SDS polyacrylamide gel electrophoresis (SDS PAGE) and gel filtration chromatography. Inhibitor-1 migrates with a molecular mass of 26,000 Da on SDS PAGE (Huang and Glinsmann, 1976) and elutes from a gel filtration column like a protein of 60,000 Da (Nimmo and Cohen, 1978). Sequence analysis show I-1 to have 165 residues and a molecular mass of 19,000 Da (Nimmo and Cohen, 1978; Aitken et al., 1982). DARPP-32 migrates with a molecular mass of 32,000 Da on SDS PAGE but has a calculated mass of 22,600 Da from sequence information (Williams et al., 1986). Similarly, inhibitor-2, migrates on SDS PAGE like a 30,000 Da protein but has 203 amino acids and a calculated molecular mass of 23,000 Da (Foulkes and Cohen, 1980; Holmes et al., 1986).

2.1.1.1 Okadaic acid, calyculin A, and microcystin

Okadaic acid, a polyether fatty acid first isolated from the marine sponge *Halichondria okadaii* and produced by dinoflagelates, is a potent,
noncompetitive inhibitor of protein phosphatases (Bialojan and Takai, 1988). PP-2A is inhibited with an IC$_{50}$ of 1 nM. PP-1 was also inhibited but with an IC$_{50}$ in the range of 100-500 nM. Okadaic acid is a much less potent inhibitor of PP-2B phosphatase with an IC$_{50}$ of 4-5 µM and does not affect PP-2C at concentrations up to 10 µM. Owing to its extreme but varied potency and relatively low cell toxicity, okadaic acid is a useful tool for determining the phosphatases involved in specific physiological events. Recently, Zhang et al. (1994) found that converting a four amino acid region in PP-1 (residues 274-277, GEFD) to the four amino acids found in the corresponding region of PP-2A (residues 267-270, YRCG) increases the sensitivity of PP-1 to okadaic acid to that of PP-2A. This amino acid sequence is thought to be involved in toxin binding.

Calyculin A, a spiro-ketal originally isolated from the marine sponge Discodermia calyx, is a very potent inhibitor of type-1 and 2A phosphatases (Kato et al., 1986). However, with nearly identical IC$_{50}$ values for PP-1 and PP-2A of 0.4 and 0.3 nM, respectively, calyculin A is not useful for distinguishing between the different phosphatases.

Cyanobacteria produce a family a toxic monocyclic heptapeptides known as the microcystins (Carmichael et al., 1988). Microcystins share a common core structure differing only in two amino acids. Two variations, microcystin-LR and microcystin-LA, are potent inhibitors of PP-1 and PP-2A activity with IC$_{50}$ values of 1.7 and 0.04 nM, respectively (Honkanen et al., 1990). This difference in potency allows microcystins to be used in the differentiation of PP-1 and PP-2A mediated events.
2.1.1.2 Other phosphatase inhibitors

NaF, ATP, and pyrophosphate are some of the molecules commonly used to inhibit protein phosphatase activity. NaF inhibits both PP-1 and PP-2A with an IC₅₀ between 1-10 mM (Resink et al., 1983; Shacter-Noiman and Chock, 1983; Waelkens et al., 1986; Bollen and Stalmans, 1988). ATP and pyrophosphate are also potent inhibitors of PP-1 and PP-2A activity (Khandelwal, 1977; Pato and Adelstein, 1983; Shacter-Noiman and Chock, 1983). Inhibition of PP-1 activity can be reversed with Mn²⁺ or Co²⁺ (Hsiao et al., 1978; Mackenzie et al., 1980; Khandelwal and Kamani, 1980; Ingebritsen et al., 1980).

2.1.2 Regulation of protein phosphatases

Traditionally phosphorylation-dephosphorylation reactions were thought to be controlled solely by the regulation of the kinase involved. This view is no longer adequate to explain the regulation of many cellular processes. Recent evidence has suggested specific regulatory mechanisms for type-1 and type-2 phosphatases. This section will describe some of the evidence for the regulation of type-1 and type-2A phosphatases.

Recently, several laboratories have described the regulation of protein phosphatases through phosphorylation. Johansen and Ingebritsen (1986) have shown that the PP-1C can be phosphorylated and inhibited by pp60⁵⁺-src tyrosine kinase. The inhibition is the result of increasing the Kₘ (Johansen and Ingebritsen 1987). The partial proteolysis of the catalytic subunit to yield a 33,000 Da species restored the phosphatase activity. The restoration of phosphatase activity correlates with the loss of the phosphorylation site. Villa-Moruzzi et al. (1991) have described the phosphorylation of PP-1G and PP-1C by v-abl tyrosine kinase. In contrast
to the report of Johansen and Ingebritsen, Villa-Moruzzi et al. do not observe inactivation of the phosphatase as a result of phosphorylation.

Villa-Moruzzi (1992) has also described the activation of PP-1 following phosphorylation of a PP-1 inhibitor-2 complex (PP-1I) by cdc2 kinase. Activation paralleled the phosphorylation of inhibitor-2 and may result from the dissociation of the PP-1I complex. Phosphorylation of the catalytic subunit by cdc2 kinase was also described but its affect on enzyme activity was not determined. Recently, Beullens et al. (1992) have shown that PP-1C can form an inactive complex with polypeptides known as nuclear inhibitors of PP-1 (NIPP-1). Subsequently, Van Eynde et al. (1994) have shown that phosphorylation of NIPP-1 by protein kinase A or casein kinase 2 restores the phosphatase activity.

It has been proposed that PP-1 activity can be regulated by heat stable inhibitor-2 (reviewed by Bollen and Stalmans, 1992; Shenolikar and Nairn, 1991). The inhibitory effect of I-2 on the catalytic subunit of PP-1 (PP-1C) has been discussed in section 2.1.1.10. PP-1C interacts with I-2 to form an inactive complex referred to as PP-1I. I-2 induces a conformational change in PP-1C leading to a loss in phosphatase activity. Phosphorylation of I-2 by glycogen synthase kinase-3 results in a restoration of the active conformation of PP-1C. Dephosphorylation of I-2 is followed by a gradual loss of phosphatase activity.

Cohen and coworkers have suggested that type-1 phosphatase activity can be regulated by directing the catalytic subunits to specific subcellular locations with specific targeting proteins (Cohen, 1990). One example of this phenomenon is the glycogen bound phosphatase known as PP-1G isolated from skeletal muscle (Strålfors et al., 1986) and liver (Wera et al., 1991). The G subunit has been identified as a 160 kDa protein
that is highly sensitive to proteolysis. The G subunit can be phosphorylated by cAMP dependent protein kinase causing the release of the PP-1 catalytic subunit from the glycogen particle (Hiraga and Cohen, 1986; Hubbard and Cohen, 1989a). The translocation of phosphatase activity from one subcellular location to another represents an important method for controlling glycogen metabolism (Hubbard and Cohen, 1989b). This subunit has been cloned from rabbit skeletal muscle cDNA. A molecular mass of 130,000 Da has been calculated from its cDNA sequence while SDS PAGE of the expressed protein suggests a protein of 160,000 Da (Tang et al., 1991). Hydropathy plots show significant hydrophobic areas capable of binding to the membrane. A similar or identical binding subunit has been suggested for directing type-1 phosphatase towards the endoplasmic reticulum (Hubbard et al., 1990).

Another form of type-1 phosphatase known as PP-1M has been purified from the myofibril fractions of skeletal and cardiac muscle (Chisholm and Cohen, 1988a and b) and chicken gizzard (Alessi et al., 1992). PP-1M is a heterotrimer composed of the PP-1 catalytic subunit and two other subunits of 130 and 20 kDa (Dent et al., 1992; Alessi et al., 1992).

Regulation of type-2A phosphatase activity has also been suggested. One mechanism of regulation is the differential expression of regulatory B subunits. Three distinct forms of the B subunit have been described (B=55 kDa, B'=54 kDa, and B''=74 kDa) as well as two isoforms of B (α and β). The B subunit isoforms are expressed in different amounts in neuronal tissue (Mayer et al., 1991). In synchronized embryonal cells the A and C subunits are expressed in greater amounts than the B subunit (Ruediger et al., 1991). Addition of the B subunits to the AC complex decreases the phosphatase activity (Imaoka et al., 1983) and increases the sensitivity
towards okadaic acid (Kamibayashi et al., 1991). Interaction of the different forms of B subunit with the AC complex produces enzymes with distinct specific activities (Usui et al., 1988). The B subunit can also be replaced with the SV-40 small-t antigen causing a decrease in phosphatase activity (Yang et al., 1991). This inhibition may play a role in the cell transformation activity of SV-40. Loss of the B subunit also affect the substrate specificity of the type 2A phosphatases. Pato and Kerc (1986) showed that proteolysis of the 55 kDa B subunit of SMP-I altered the substrate specificity such that the phosphatase became active towards intact myosin.

Recently, Chen et al. (1992) have shown that p60v-src, p56lck, the epidermal growth factor receptor, and the insulin receptor can phosphorylate tyrosine 307 of the PP-2A catalytic subunit in vitro resulting in a 90% inhibition of phosphatase activity. Phosphorylation can be enhanced with the addition of okadaic acid to inhibit autodephosphorylation. The physiological importance of PP-2A phosphorylation has not been shown.

2.2 Smooth muscle contraction

The mechanical nature of smooth muscle contraction and the regulation of the contractile process has been the subject of intense study for many years. The protein composition of the smooth muscle contractile apparatus has been determined. Fluctuations in the free cytosolic calcium concentration ([Ca^{2+}]_i) have been identified as the primary regulatory signal for smooth muscle contraction. Increased [Ca^{2+}]_i stimulates the phosphorylation of the 20,000 Da myosin light chains by myosin light chain kinase resulting in muscle contraction.
Dephosphorylation catalyzed by a myosin phosphatase relaxes the muscle. Secondary calcium linked regulatory processes have been suggested in the modulation of smooth muscle contractile activity. The following sections will review the mechanism of smooth muscle contraction and the proteins important in the contractile process.

2.2.0 Structure of smooth muscle

The smooth muscle contractile apparatus is composed of interdigitated filaments held in place by a latticework of fibers and dense

![Diagram of smooth muscle cell structure](image)

**Figure 2.1.** Schematic representation of a smooth muscle cell. Panel 1 shows the arrangement of intermediate filaments and dense bodies within the smooth muscle cell. Panel 2 shows the contractile units composed of myosin (thick filaments) and actin (thin filaments). This figure was modified from a diagram in *The Structural Basis of Muscle Contraction* by J. Squire (p. 459), published by Plenum Press (1981), used with permission.
bodies (figure 2.1). The main contractile filaments are referred to as thick
and thin filaments owing to their appearance under the light microscope.
The composition of the thick and thin filaments as well as the supporting
lattice are discussed in the following sections.

2.2.0.1 Thin filaments

The thin filaments, with a diameter of 4-7 nm, (Nonomura, 1968)
are composed mainly of filamentous actin (F-actin) (Hanson and Lowy,
1963). Thin filament actin attaches to both the cytoplasmic and membrane
bound dense bodies that form the anchors for smooth muscle contraction
(Ashton et al., 1975). Associated with actin in the thin filament are
tropomyosin, caldesmon, and calponin. In contrast to skeletal and cardiac
muscle thin filaments, the troponin complex has not been identified in
smooth muscle.

2.2.0.1.0 Actin

Actin is a highly conserved protein present in both muscle and
non-muscle cells. Cytosolic or G-actin is a globular protein of 42,000 Da
which can polymerize to form a double-stranded helical structure known
as F-actin. F-actin forms the backbone of the muscle thin filament.
Studies using isoelectric focusing gel electrophoresis have revealed three
actin isoforms, α, β, and γ, with identical molecular weights (Whalen et
al., 1976). Amino acid sequencing of the actin isoforms has uncovered
differences in at least 25 residues. The different isoelectric points of these
isoforms are caused by changes in three acidic residues at the N-terminus
(1977) have found that the most acidic isoform, α actin, co-migrates with
the skeletal muscle actin while the least acidic γ isoform co-migrates with the smooth muscle actin. *In vitro* studies have shown little functional difference between skeletal and smooth muscle actin. All three forms of actin can be present in the same cell type in varied amounts (Elce *et al.*, 1981). A detailed analysis of turkey gizzard actin found that 75 % of the protein is of the γ isoform while 25 % is β non-muscle cytosolic actin (Rubenstein, 1981). Fatigati and Murphy (1984) have studied the actin composition in a range of smooth muscle types and found a tissue specific inverse relationship between α and γ actin. β non-muscle actin was found in all cell types and may be involved in secretion, cell proliferation or other functions not directly related to the contractile process. A recent study has localized the different actin isoforms within the smooth muscle cell. Using β-specific antibodies the cytoplasmic form of actin has been localized to the dense bodies and thin filaments (North *et al.*, 1994) while the smooth muscle γ form of actin is limited to the contractile apparatus.

### 2.2.0.1.1 Tropomyosin

Tropomyosin is an asymmetric dimer formed from two 33,000 Da α-helical subunits which wrap around one another in a coiled-coil. At low ionic strength, tropomyosin is able to form end to end polymers that fit into the grooves between the actin α-helices. It binds to actin with a stoichiometry of 1 tropomyosin molecule for each 7 actin molecules (Murray and Weber, 1974). Structural studies have uncovered several isoforms of skeletal and cardiac tropomyosin (Cummins and Perry, 1973; Cummins and Perry, 1974; Leger *et al.*, 1976; Cummins, 1979). Two isoforms of tropomyosin have since been described for smooth muscle (Driska and Hartshorne, 1975; Yamanaguchi *et al.*, 1984; Fatigati and Murphy,
Sequence analysis described two 284 amino acid isoforms, \( \gamma \) and \( \beta \), with a high degree of homology to the skeletal muscle \( \alpha \) and \( \beta \) isoforms, respectively (Ruiz-Opazo et al., 1985; Lau et al., 1985; Sanders and Smillie, 1985). Although the tropomyosin isoforms are of identical molecular weight (33,000) they migrate on SDS PAGE with apparent Mr of 42,000 (\( \gamma \)) and 38,000 (\( \beta \)). Several studies have shown that tropomyosin can stimulate the actin-activated myosin ATPase activity of smooth muscle myosin (Sobieszek and Small, 1977). This stimulation is dependent on calcium concentration (Nag and Seidel, 1983) and ionic strength (Williams et al., 1984). However, the function of tropomyosin in smooth muscle contraction remains poorly understood.

### 2.2.0.1.2 Caldesmon

Caldesmon, first identified as a 130,000 Da protein associated with the thin filaments of chicken gizzard of smooth muscle (Driska and Hartshorne, 1975; Sobue et al., 1981) has subsequently been identified in the smooth muscle of turkey gizzard (Malencik et al., 1989) bovine aorta, uterus and human platelets (Kakiuchi et al., 1983; Clark et al., 1986). A second class of caldesmon in the range of 71,000 to 77,000 Da has been identified in chicken and turkey gizzards (Malencik et al., 1989; Bretscher and Lynch, 1985). Caldesmon binds to F-actin with a stoichiometry ranging from 1 mole caldesmon : 10 moles of actin (Clark et al. 1986) to 1:28 moles actin (Smith et al., 1987; Graceffa et al., 1988). The interaction of caldesmon with actin is independent of calcium while binding to calmodulin is dependent on both calcium and ionic strength (Sobue et al., 1981; Kakiuchi et al., 1983; Malencik et al., 1989). The actin and calmodulin binding sites reside in the C-terminus of caldesmon (Szpacenko and
Dabrowski, 1986) while the 12 amino terminal residues of actin have been implicated in the binding of caldesmon (Bartegi et al., 1990). It is interesting to note that these are the same residues involved in activation of the myosin ATPase activity (Sutoh, 1982). Caldesmon has been shown to bind tropomyosin *in vitro* (Graceffa, 1987; Smith et al., 1987, Horiuchi and Chacko, 1988) while *in situ* hybridization has localized caldesmon to the stress fibers where it shows the same periodicity as tropomyosin (Bretsher and Lynch, 1985). Ikebe and Reardon (1988) showed that caldesmon binds to the subfragment 2 region of myosin and that binding can be abolished by Ca$^{2+}$-calmodulin. The myosin binding site has been localised to the N-terminal region of caldesmon (Velaz et al., 1990).

2.2.0.1.3 Calponin

Calponin is a 34 kDa calcium-calmodulin and filamentous actin binding protein (Takahashi et al., 1986) that has been identified in bovine aorta, esophagus, stomach, trachea and uterus as well as the non-muscle tissues adrenal medulla and cortex (Takahashi et al., 1987). Isoelectric focusing gel electrophoresis has been used to identify several calponin isoforms in bovine aorta and chicken gizzard (Takahashi et al., 1988). Calponin shares some properties with troponin T. It cross-reacts with antibodies to the C-terminal portion of troponin T and binds to troponin C (Takahashi et al., 1988). Nishida et al. (1990) have shown a stoichiometry of 7 actin : 0.9 tropomyosin : 0.6 caldesmon : 0.7 calponin. The binding of calponin to actin has been localized to the N-terminal region between residues 52-168 while the binding site on actin is at the C-terminus between residues 326-355 (Mezgueldi et al., 1992). Makuch et al. (1991) have shown that both caldesmon and calponin can bind thin
filaments when concentrations of both are sub-saturating, if the concentration of either is increased, the other is displaced from the thin filament.

2.2.0.2 Thick filaments

Myosin is the major component of the thick filaments. Other components such as C-protein have been identified in the thick filaments of striated muscle (Murphy and Megerman, 1977). The minor components of smooth muscle thick filaments are not well studied. Thick filaments are formed from polymers of myosin whose C-terminal 80 nm segments intertwine to form the filament backbone with the remainder of the rod-like portion and the globular heads protruding outward to form the crossbridge with actin (Huxley, 1963). The myosin molecules at one end of the filament are arranged with the opposite polarity with respect to the molecules at the other end. Thick filaments are arranged in bundles surrounded by many actin filaments and run along the long axis of the smooth muscle cell (Kelly and Rice, 1968). The ratio of thin to thick filaments in smooth muscle is at least 12:1 (Devine and Somlyo, 1971), which is higher than that of striated muscle. Despite the higher thin to thick filament ratio, smooth muscles generate about the same level of force as striated muscles. The level of force development in smooth muscle may be explained by the length of the filaments themselves. Thick filaments from smooth muscle are 2.2 μm long in contrast to the thick filaments of skeletal muscle at 1.6 μm in length (Ashton et al., 1975).
2.2.0.2.0 Myosin

Myosin is a large hexameric protein composed of two identical heavy chains and two pairs of light chains (figure 2.2) (reviewed by Harrington and Rodgers, 1984; Warrick and Spudich, 1987; Hartshorne, 1987). Each 200,000 Da heavy chain has an extended \( \alpha \)-helical domain at the C-terminus and a globular head at the N-terminus. The \( \alpha \)-helical domains wrap around one another to form a coiled-coil. The two pairs of light chains (20 and 17 kDa) are associated with the head region. The myosin molecule can be divided into several structural and functional domains. Myosin contains two proteolytic sensitive sites (1 and 2). Proteolysis at site 1 cleaves myosin into a C-terminal segment designated as light meromyosin (LMM) and an N-terminal portion containing the remaining coiled-coil and the globular heads designated heavy meromyosin (HMM). The LMM is involved in the polymerization of myosin into thick filaments and is insoluble in low ionic strength buffer. HMM, which is moderately soluble, retains a functional ATPase and the actin binding site. These properties allows HMM to be used for many in vitro experiments where the poorly soluble myosin is unsuitable. A second proteolytic site fractionates HMM into subfragment 1 (S-1) and subfragment 2 (S-2). The S-1 fragment comprises the myosin head region which retains the \( \text{Mg}^{2+} \)-ATPase and actin binding site while the S-2 fragment comprises the remainder of the coiled-coil. Recently, the three dimensional structure of S-1, solved by X-ray crystallography, has shown that the actin binding site and the ATPase active site are located on opposite sides of the fragment (Rayment et al., 1993a). The two sites are separated by a cleft that splits the axial one third of the fragment into two domains.
Figure 2.2. A schematic representation of the myosin molecule. The structure of myosin and its proteolytic products are represented. This figure was modified from a diagram in Biochemistry of the Contractile Process in Smooth Muscle by D. J. Hartshorne (p 431) from Physiology of the Gastrointestinal Tract edited by L. R. Johnson, published by Raven Press (1987), used with permission.
The two pairs of myosin light chains (MLC) bind non-covalently to the junction between the S-1 and S-2 fragments (Wilkelmann et al., 1983; Flicker et al., 1983). Sellers and Harvey (1984) have further localized light chain binding to a 26,000 Da segment at the C-terminus of the S-1 fragment. The three dimensional crystallographic structure of the S-1 fragment has confirmed the spacial relationship of the myosin light chains to the myosin head (Rayment et al., 1993). The myosin light chains are involved in the contractile process. The 20,000 Da myosin light chains are phosphorylated in the Ca$^{2+}$-dependent activation of smooth muscle contraction (discussed in section 2.2.2.1) whereas the 17,000 Da myosin light chains are required for the maintenance of Mg$^{2+}$-ATPase activity.

2.2.0.3 Dense bodies and intermediate filaments

Microscope studies of smooth muscle have shown opaque structures distributed throughout the cytoplasm and attached to the plasma membrane. The membrane bound plaques are enriched with vinculin, a protein with a molecular mass of 130,000 Da, which links actin containing thin filaments to the cell membrane (Geiger et al., 1980, 1981). The dense bodies, enriched with the 110,000 Da protein $\alpha$-actinin, provide an anchor for the thin filament within the cytoplasm (Schollmeyer et al., 1976; Fay et al., 1983; Small, 1985). Intermediate filaments form a scaffold within the smooth muscle cell to which the dense bodies are attached (Cooke and Fay, 1972; Campbell et al., 1979). The intermediate filament, composed of the 55,000 Da protein desmin, does not participate directly in the contractile process (Cooke, 1976; Small and Sobieszek, 1977; Lazerides, 1980). Fay et al. (1983) have shown that the distance between the dense bodies changes with the level of muscle contraction suggesting that as the
actomyosin slides, it pulls the dense bodies along thereby changing the length of the cell. Dense bodies may be the smooth muscle correlate of the skeletal muscle Z line. In support of this idea, Bond and Somlyo (1982) have demonstrated that dense bodies bind actin with a polarity similar to that seen in the binding of actin to the skeletal muscle Z-line. In this study the S-1 fragment of myosin was added to a preparation of filamentous actin that was anchored at one end to dense bodies. An arrowhead structure pointing away from the dense bodies was formed when S-1 bound to actin illustrating the polarity of the actin filament.

2.2.1 Mechanism of smooth muscle contraction

In 1954, Huxley and Hanson (1954) and Huxley and Niedergerke (1954) independently proposed the sliding filament model for muscle contraction. This model involves the arrangement of the muscle cell into contractile units called sarcomeres composed of thick and thin filaments (figure 1.1). Structural proteins anchor the thin filaments at either end. Upon stimulation, the filaments form cross-bridges and slide past one another pulling the contractile unit along and shortening the muscle cell. Coordination of this process causes muscle contraction. Although originally used to describe contraction in striated muscle this model also applies to smooth muscle.

2.2.1.0 Cross-bridge cycling

The contractile unit in smooth muscle is composed of actin and myosin filaments. Contraction occurs when these filaments slide past one another. This movement is mediated by the process of cross-bridge cycling (reviewed by Adelstein and Eisenberg, 1980; Cooke, 1986). The cross-bridge
is produced through the interaction of the myosin head with one molecule of actin within the thin filament. The different steps in this process are described in figure 2.3.

Cross-bridge cycling is initiated by the phosphorylation of myosin by myosin light chain kinase (discussed in section 2.2.2.1). Phosphorylated myosin is able to interact with actin. The actin-myosin complex binds ATP thereby reducing the affinity of actin for myosin. The dissociation of the actin-myosin complex is followed by the hydrolysis of ATP to ADP and Pi (step 1). The myosin-ADP+Pi complex has an increased affinity for actin and readily reforms the actin-myosin complex (step 1). The release of Pi is associated with a conformational change in the myosin head (step 2). This event, known as the power stroke, provides the mechanical energy of muscle contraction. The final step (step 3) involves the exchange of ADP for ATP and the return of myosin to its resting conformation.

2.2.1.1 Latch state

Smooth muscle contraction occurs in response to a transient increase in free cytosolic calcium concentration ([Ca$^{2+}$]$_i$) (section 2.2.2.0). Relaxation normally follows the removal of calcium from the sarcoplasm. Under certain circumstances tension is maintained after [Ca$^{2+}$]$_i$ has decreased. This phenomenon was first observed by Dillon et al. (1983) who reported the maintenance of tension in arterial smooth muscle even after a decrease in [Ca$^{2+}$]$_i$ and the dephosphorylation of MLC$_{20}$. This phenomenon was termed latch owing to its similarity to the catch state of molluscan muscle. Subsequently, low levels of MLC$_{20}$ phosphorylation
Figure 2.3. Model for cross-bridge cycling. Panel 1 shows the hydrolysis of ATP to ADP and $P_i$. Panel 2 shows the release of $P_i$ and the formation of a strained crossbridge. Panel 3 shows the exchange of ATP for ADP and the return to the unstrained crossbridge. This figure was modified from a diagram in Biochemistry of the Contractile Process in Smooth Muscle by D. J. Hartshorne from Physiology of the Gastrointestinal Tract (p 464) edited by L. R. Johnson, published by Raven Press (1987), used with permission.

and slow cross-bridge cycling were shown to occur during latch (Ratz et al., 1989). A simple four step kinetic model (figure 2.4) has been proposed to
explain latch (Murphy 1994). Central to this model is the dephosphorylation of the 20,000 Da myosin light chains while myosin is actively forming a cross-bridge with actin, as described by $K_5$ (panels 3 and 4, figure 2.4). The dephosphorylated actomyosin has a much decreased rate of ATP hydrolysis ($K_7$) accounting for the decreased ATP consumption observed during latch. The unphosphorylated actomyosin complex described in panel 4 is a substrate for myosin light chain kinase, however, the rate of this phosphorylation ($K_6$) is much lower than the rate of phosphorylation of actin-free myosin ($K_1$). Although the calcium levels are low, sufficient calcium concentration is present to produce sub-maximal activation of the myosin light chain kinase. This results in the rephosphorylation of the 20,000 Da myosin light chains and therefore reformation of the cross-bridge ($K_6$). The phosphorylation described by $K_6$ allows for low levels of cross-bridge cycling to occur during the latch state. Murphy suggests that latch can occur when the myosin phosphatase activity is elevated with respect to the myosin light kinase activity allowing for the dephosphorylation of myosin attached to actin.

2.2.1.2 Smooth muscle energetics

The energy required for smooth muscle contraction is provided by the hydrolysis of ATP which is catalysed by the actin activated $\text{Mg}^{2+}$ ATPase of the myosin head (Paul, 1989). ATP consumed in the phosphorylation of the myosin regulatory light chains does not contribute significantly to smooth muscle energy consumption. ATP utilization increases with force development. In vascular smooth muscle a three fold increase in ATP utilization has been observed during contractile activity. The steady state ATP utilization and force development depend on the
length of the contractile fibers, the load on the muscle, and on the intrinsic ATPase activity. Maximum rate of energy consumption occurs during muscle shortening. Force maintenance requires less energy than active contraction. As fibers approach their minimum length the ATP

Figure 2.4. A four step cross-bridge model describing smooth muscle contraction and latch. Panels 1 and 2 show the phosphorylation of myosin. Crossbridge cycling is shown in panels 2 and 3. The latch state is illustrated in panel 4. This figure modified from a diagram in What is special about smooth muscle? The significance of covalent crossbridge regulation by R.A.Murphy in FASEB J. 8: 311-318, 1994. Used with permission.
utilization decreases to 20-50% of the suprabasal levels (Butler and Seigman, 1985). In contrast to skeletal muscle, which depends on a pool of high energy phosphate to maintain ATP levels, tonic smooth muscle relies extensively on aerobic metabolism for its ATP production. Phasic smooth muscle falls between these extremes relying mainly on aerobic metabolism but also utilizing phosphocreatine to maintain its ATP levels (Hellstrand and Paul, 1983).

2.2.2 Regulation of smooth muscle contraction

The principal regulatory mechanism in muscle contraction is the free intracellular calcium concentration ([Ca$^{2+}$]$_i$). Calcium is integral to the regulation of skeletal, cardiac and smooth muscle contraction. However, the mechanisms of this regulation differ for the different muscle types. The following section describes the central role of calcium and the regulatory mechanisms specific to smooth muscle contraction.

2.2.2.0 Calcium

It is widely accepted that smooth muscle contraction which occurs in response to electrical stimulus, K$^+$ depolarization or excitatory agonists is mediated through an increase in the free intracellular calcium concentration ([Ca$^{2+}$]$_i$) (reviewed by Bolton, 1979; Somlyo and Himpens, 1989). The calcium concentration in resting smooth muscle ranges between 80 and 270 nM while an increase in the calcium concentration to 500-700 nM results in contraction (Williams and Fay, 1986; Somlyo and Himpens, 1989, DeFeo and Morgan, 1985; Walsh, 1991). In mammals, calcium is found within intracellular compartments and in the extracellular fluids. The sarcoplasmic reticulum (SR) is the main storage
depot for cellular calcium (Somlyo and Somlyo, 1971; Devine et al., 1972, Somlyo et al., 1979). Release of calcium from the sarcoplasmic reticulum is stimulated by inositol trisphosphate (IP$_3$) which binds to a specific receptor located on the SR membrane (Somlyo, 1985; Iino, 1987; Chadwick et al., 1990). Repeated IP$_3$ stimulation results in repeated release of calcium (Iino, 1987). Extracellular calcium enters the sarcoplasm through both receptor operated and voltage-dependent calcium channels located on the plasma membrane (Hurwitz, 1986). In most smooth muscle types extracellular calcium alone is insufficient to produce the threshold concentration required for contraction; however, extracellular calcium will stimulate calcium-induced calcium release from the sarcoplasmic reticulum and result in contraction (Iino et al., 1988). Normally, sequestration of intracellular calcium into the sarcoplasmic reticulum results in relaxation.

2.2.2.1 Myosin linked regulation- myosin light chain phosphorylation

The myosin linked system for the regulation of smooth muscle contraction was first suggested by Bremel in 1974. Bremel examined the actin activated Mg$^2+$-ATPase activity of smooth muscle myosin, the in vitro correlate of muscle contraction, in the presence of pure skeletal muscle filamentous actin. Under these conditions ATPase activity was observed only after the addition of calcium. Bremel concluded that calcium was necessary for smooth muscle contraction and that the regulatory mechanism was associated with the smooth muscle myosin. A subsequent study by Sobieszek and Small (1976) supported this conclusion. This study showed that the interaction of smooth muscle thin filaments with skeletal muscle myosin produced an actomyosin that did not require
calcium for activation of its ATPase activity indicating that the smooth muscle thin filaments do not contain calcium sensitive regulatory elements.

Sobieszek was the first to suggest that the myosin linked regulatory mechanism for smooth muscle contraction was mediated through the phosphorylation of the 20,000 Da myosin light chains (MLC$_{20}$) by a specific calcium dependent protein kinase (Sobieszek, 1977; Bremel et al., 1977). Hartshorne and co-workers subsequently demonstrated that phosphorylation of MLC$_{20}$ by myosin light chain kinase is a key regulatory step in the Ca$^{2+}$-dependent activation of the actin-activated myosin-Mg$^{2+}$ATPase activity (Askoy et al., 1976; Gorecka et al., 1976). Using purified myosin, Chacko et al. (1977) demonstrated that the actomyosin ATPase activation through phosphorylation could be reversed by subsequent dephosphorylation of myosin. Sellers et al. (1981) confirmed these results using purified proteins in a reconstituted actomyosin system. The actin-activated myosin-Mg$^{2+}$ATPase activity of isolated myosin and heavy meromyosin was quantitated through a cycle of phosphorylation, dephosphorylation and rephosphorylation. The actin-activated myosin-Mg$^{2+}$ATPase activity increased from 4 to 51 nmol/min/mg with phosphorylation and returned to 5 nmol/min/mg with dephosphorylation. Rephosphorylation restored the high ATPase activity to 46 nmol/min/mg. HMM showed a similar pattern, albeit with elevated specific activities, going from 10 nmol/min/mg when unphosphorylated to 357 nmol/min/mg with phosphorylation and returning to 20 nmol/min/mg upon dephosphorylation. The elevated ATPase activity was restored to 371 nmol/min/mg with rephosphorylation. This study showed that actin, myosin, myosin light chain kinase and a myosin
phosphatase were sufficient to regulate the actin-activated myosin-
Mg\(^{2+}\)-ATPase activity.

Using skinned smooth muscle fibers, Hoar et al. (1979) have
correlated the calcium dependent MLC\(_{20}\) phosphorylation with tension
development. These researchers observed that the 20,000 Da myosin light
chains were the only proteins to be significantly phosphorylated following
incubation of skinned muscle fibers with Ca\(^{2+}\) or Sr\(^{2+}\). Thiophosphorylation of MLC\(_{20}\) using ATP\(\gamma\)S resulted in an irreversible
contraction. The calcium dependence of MLCK was underlined in a study
by Kerrick et al. (1981) where calmodulin antagonists were shown to
impede tension development in skinned smooth muscle. Furthermore,
addition of a calcium-insensitive form of MLCK to skinned smooth
muscle resulted in force development in the absence of calcium (Walsh et
al., 1982). Tension produced in skinned smooth muscle can be reversed
with incubation with the catalytic subunit of PP-2A (Haeberle et al., 1985)
or the myosin phosphatase SMP-IV (discussed in section 2.2.2.3) (Hoar et
al., 1985). Furthermore, smooth muscle contracted through the
thiophosphorylation of myosin remain contracted even after the addition
of the catalytic subunit of PP-2A (Haeberle et al., 1985).

Myosin light chain phosphorylation was also shown to correlate
with tension development in intact smooth muscle. DeLanerolle and
Stull (1980) induced contraction in tracheal smooth muscle by incubation
with methacholine, a reagent which increases the [Ca\(^{2+}\)]\(_i\). Increased
tension was correlated with an increase in MLC\(_{20}\) phosphorylation.
Subsequent relaxation with atropine caused a decrease in MLC\(_{20}\)
phosphorylation. Driska et al. (1981) confirmed these results using arterial
smooth muscle stimulated with KCl. An increase in MLC\(_{20}\)
phosphorylation was found to precede force development. These observations provide strong evidence for the regulation of smooth muscle contraction through the reversible phosphorylation of myosin.

The site of MLC$_{20}$ phosphorylation has been elucidated. Amino acid sequence analysis of MLC$_{20}$ phosphopeptides has identified serine 19 as the primary phosphorylation site (Jakes et al. 1977; Pearson et al., 1984). High MLCK levels, extended incubation times, and low ionic strength result in the diphosphorylation of MLC$_{20}$ at threonine 18 and serine 19 (Cole et al., 1985; Ikebe and Hartshorne, 1985). The physiological relevance of the threonine 18 phosphorylation is uncertain.

2.2.2.2 Myosin light chain kinase

Phosphorylation of the 20,000 Da myosin light chains (MLC$_{20}$) is catalyzed by myosin light chain kinase (MLCK). Myosin light chain kinase was first purified from chicken gizzard as a heterodimer of 130,000 and 17,000 Da proteins (Dabrowska et al., 1977). The 17,000 Da component was shown to be the calcium binding protein calmodulin (Dabrowska et al., 1978). Subsequently, the catalytic subunit was isolated as a 130,000 molecular weight protein which required calcium and calmodulin for activity and was highly specific for the 20,000 Da regulatory light chains of myosin (Adelstein and Klee, 1981). The amino acid sequence deduced from the cDNA of the chicken gizzard enzyme shows MLCK to be a protein of 107 kDa (Guerriero et al. 1986, Olson et al., 1990). The calmodulin binding domain has been localized to a C-terminal peptide comprising amino acids 796-813.

Activation of MLCK requires the formation of a complex with calcium-calmodulin. Calmodulin has four high affinity calcium binding
sites. At least three of these sites must be occupied for activation to occur (Kamm and Stull, 1985). A pseudosubstrate domain has been identified at the C-terminus of the MLCK molecule which overlaps with the calmodulin-binding region (Foyt et al., 1985; Walsh, 1985; Pearson et al., 1988). In the absence of calcium-calmodulin, MLCK assumes a conformation whereby the pseudosubstrate domain interacts with the active site inhibiting the enzyme activity. The formation of a calcium-calmodulin-MLCK complex induces a conformational change that dissociates the pseudosubstrate domain from the active site and activates the enzyme. Partial proteolysis of MLCK cleaves the calcium-calmodulin binding region and the pseudosubstrate domain resulting in a non-regulated enzyme (Ikebe et al., 1987).

Myosin light chain kinase activity can be regulated through phosphorylation. Adelstein et al. (1978) showed that MLCK can be phosphorylated in vitro at two sites, A and B, by the catalytic subunit of cAMP-dependent protein kinase resulting in a decrease in affinity of MLCK for calcium-calmodulin (Conti and Adelstein, 1981). Sites A and B have been identified as serine 815 and serine 828, respectively (Lukas et al., 1986; Payne et al., 1986). Calcium-calmodulin inhibits the phosphorylation of site A but has no effect on the phosphorylation at site B suggesting that site A and the calmodulin binding site are in close proximity (Conti and Adelstein, 1981).

The physiological relevance of the phosphorylation of MLCK is still a matter of debate. Studies using isolated actomyosin, skinned smooth muscle fibers, and intact smooth muscle have been used to correlate a decrease in calcium sensitivity with cAMP-dependent protein kinase activity. The calcium sensitivity of bovine aortic actomyosin actin-
activated myosin Mg\(^{2+}\)-ATPase activity decreases following incubation with cAMP-dependent protein kinase (Silver et al., 1981). Tension development in skinned muscle fibers from chicken gizzard (Kerrick and Hoar, 1981), guinea pig taenia coli (Ruegg et al., 1981), and porcine carotid artery (Ruegg and Paul, 1982) showed a decreased calcium sensitivity following incubation with the catalytic subunit of cAMP-dependent protein kinase. This effect was antagonized by high levels of calcium and calmodulin (Kerrick and Hoar, 1981; Meisher and Ruegg, 1983). Intact smooth muscle treated with forskolin, a reagent known to increase cAMP levels, results in relaxation (DeLanerolle et al., 1984). Relaxation coincides with an increase in the phosphorylation of MLCK supporting the notion that PK-A can mediate relaxation.

Indeed isoproterenol, a β-adrenergic agonist, is known to stimulate relaxation of intact smooth muscle (Diamond, 1978). However, a recent study by Stull et al. (1990) has shown that MLCK is not phosphorylated in response to isoproterenol. Furthermore, Miller et al. (1983) have shown that MLCK activity does not change in intact smooth muscle following isoproterenol stimulated relaxation. In view of this information a physiological role for the PK-A phosphorylation of MLCK remains in doubt.

Recent studies suggest that MLCK might be regulated by phosphorylation by calcium-calmodulin dependent kinase II instead of PK-A. Calcium-calmodulin dependent kinase II also phosphorylates sites A and B on MLCK in vitro (Ikebe and Reardon, 1990) decreasing its affinity for calcium-calmodulin (Hashimoto and Soderling, 1990). Incubation of intact tracheal smooth muscle with KCl or carbachol results in the phosphorylation of site A (Stull et al., 1990) Smooth muscle cells
stimulated with ionomycin show an increase in \([\text{Ca}^{2+}]_i\) and, subsequently, an increase in the level of both MLC\(_{20}\) and MLCK phosphorylation (Tansey et al., 1992). Addition of KN-62, a specific inhibitor of calcium-calmodulin dependent kinase II, inhibits MLCK phosphorylation and decreases the calcium sensitivity of cell lysates. Using permeabilized smooth muscle, the \([\text{Ca}^{2+}]_i\) required for the half maximal phosphorylation of MLCK was shown to be 500 nM whereas that required for MLC\(_{20}\) phosphorylation was 250 nM (Tansey et al., 1994). Addition of KN-62 to this system abolished the phosphorylation of MLCK and decreased the \([\text{Ca}^{2+}]_i\) required for the half maximal phosphorylation of MLC\(_{20}\) to 170 nM. These data suggest that the most likely physiological regulator of MLCK activity is calcium-calmodulin dependent kinase II.

2.2.2.3 Myosin phosphatase

Smooth muscle relaxation depends on the dephosphorylation of the 20,000 Da myosin light chains (MLC\(_{20}\)). The exact nature of the phosphatase involved is still under investigation. A number of phosphatases with activity towards myosin light chains have been described. Pato and co-workers have identified four myosin light chain phosphatases termed smooth muscle phosphatases (SMP) -I, -II, -III, and -IV in cytosolic extracts of turkey gizzard smooth muscle. SMP-I, a heterotrimeric (60, 55, and 38 kDa subunits) type 2A phosphatase dephosphorylates the isolated MLC\(_{20}\) but is inactive towards intact myosin (Pato and Adelstein, 1983a; Pato et al., 1983). Therefore, it is unlikely to be directly involved in the relaxation process. SMP-II is a type 2C myosin light chain phosphatase which is also unable to dephosphorylate intact myosin (Pato and Adelstein, 1983b; Pato et al., 1983). Thus, it is not likely to
play a direct role in the relaxation process. SMP-III and SMP-IV are both active towards MLC$_{20}$ and intact myosin and may be directly involved in the in vivo dephosphorylation of myosin (Pato and Adelstein, 1983a, Pato and Kerc, 1985). This idea is supported by the observation of Hoar et al. (1985) who demonstrated that SMP-IV can relax skinned muscle fibers that have been precontracted with calcium insensitive MLCK.

Other myosin light chain phosphatases have also been described. Morgan et al. (1976) have identified a 70 kDa myosin light chain phosphatase in rabbit skeletal muscle. The activity of this enzyme towards intact myosin was not determined. The relationship of this enzyme to the smooth muscle myosin phosphatases is unknown.

Werth et al. (1982) have purified a heterodimeric phosphatase (67 and 38 kDa subunits) from bovine aorta. This enzyme has a broad substrate specificity but shows the highest specific activity towards MLC$_{20}$ and intact myosin. The subunit structure of this enzyme is reminiscent of the dimeric form of PP-2A and thus may be related to SMP-I. Pato and Kerc (1986) have shown that limited proteolysis preferentially degrades the 55 kDa subunit of SMP-I resulting in a dimeric enzyme with activity towards intact myosin.

Onishi et al. (1982) have purified a heterotrimeric phosphatase (67, 54, and 38 kDa subunits) from chicken gizzard. This enzyme was shown to inhibit the actomyosin ATPase and prevents the ATP-induced superprecipitation of actomyosin by dephosphorylation of MLC$_{20}$.

Mumby et al. (1987) have described two enzymes from bovine heart termed PT-1 and PT-2 with activity towards intact cardiac myosin. PT-1 is a heterotrimer of 63, 55 and 38 kDa while PT-2 is a dimer composed of 63 and 38 kDa subunits identical to those of PT-1. The subunit composition is
like that of SMP-I and the phosphatases described by Werth, Onishi and coworkers. The exact relationship of these enzymes remains unclear.

A phosphatase has been isolated from bovine aorta that is active towards isolated cardiac myosin light chains (DiSalvo et al., 1983). This enzyme dephosphorylates myosin light chains in a preparation of aortic actomyosin and reduces the rate of actomyosin superprecipitation. The subunit composition of this enzyme was not described.

Other phosphatases have recently been isolated from the myofibril fraction of smooth muscle. Alessi et al. (1992) described a heterotrimer with 20 and 130 kDa regulatory subunits and a 37 kDa catalytic subunit from chicken gizzard. This enzyme has been classified as a type-1 phosphatase and has been termed PP-1M due to its myofibril binding properties. Okubo et al. (1993) have isolated a phosphatase termed MBP from chicken gizzard composed of 58 and 38 kDa subunits. The myosin-binding properties have been attributed to the 58 kDa subunit which may be a proteolytic product of a 130 kDa protein (Okubo et al., 1994). The MBP catalytic subunit has been described as a type-1 phosphatase based on its cross-reactivity with type-1 specific antibodies. The smooth muscle myosin associated phosphatase (MAPP) from chicken gizzard described by Mitsui et al. (1992) is a tetramer of 34 kDa subunits. MAPP has been characterized enzymatically as a type-1 phosphatase. The myosin binding properties of this enzyme were not described. A monomeric myosin phosphatase (Mr 35,000) has recently been isolated from turkey gizzard smooth muscle myofibril (Nowak et al., 1993). Similar to SMP-III and -IV, this enzyme can not be classified as type-1 or type-2 on the basis of enzymatic data. Although capable of binding myosin, this phosphatase
has a much lower binding constant than those described for other myosin phosphatases (Sellers and Pato, 1984; Okubo et al., 1993).

### 2.2.3 Secondary regulation

The principal regulatory mechanism for smooth muscle contraction is the calcium-mediated reversible phosphorylation of the 20,000 Da regulatory light chain. Recent observations have suggested that the level of smooth muscle contraction can be modulated by secondary regulatory mechanisms. Four of these mechanisms are reviewed in this section.

### 2.2.3.0 Regulation by GTP

One proposed mechanism for regulating contraction is through GTP and GTP-binding proteins. Using permeabilized smooth muscle, it was shown that the calcium sensitivity of the contractile process could be modulated by the addition of norepinephrine and GTP-γ-S (Nishimura et al., 1988). This observation suggested a role for a G-protein mediated cascade in smooth muscle contraction. Subsequently, several groups have demonstrated an increase in smooth muscle calcium sensitivity using micromolar amounts of GTP-γ-S or GTP (Fujiwara, 1989; Kitazawa et al., 1989; Kitazawa et al., 1991a, 1991b). The increase in calcium sensitivity has been speculated to occur through the inhibition of the myosin phosphatase. Stull and co-workers subsequently demonstrated the inhibition of myosin phosphatase activity in permeabilized smooth muscle extracts treated with GTP-γ-S (Kubota, 1992).

The mechanism of action for GTP is still unclear. Several groups have suggested that GTP activates a trimeric G-protein resulting in a cascade that terminates with the inactivation of the myosin phosphatase.
In support of this conclusion Hai and Ma (1993) used fluoroaluminate, a G-protein specific agonist, to demonstrate an increase in calcium sensitivity. In the absence of calcium, fluoroaluminate treatment resulted in myosin light chain phosphorylation and stress in bovine trachea.

Small monomeric cytosolic GTP-binding proteins have also been implicated in the GTP-induced increase in calcium sensitivity. rho p21 is one such protein shown to affect the contractile state of smooth muscle. Hirata et al. (1992) showed that ADP-ribosylation of rho p21 abolished the GTP-γ-S induced increase in calcium sensitivity. The calcium sensitivity was restored by the addition of rho p21 complexed with GTP-γ-S. H-ras p21 has also been implicated in the modulation of calcium sensitivity in smooth muscle. Skinned smooth muscle incubated with the GTP-bound form of H-ras showed increase in force production at submaximal calcium concentration (Satoh et al., 1993).

2.2.3.1 Phosphorylation by protein kinase C (PK-C)

Protein kinase C is a broad-specificity diacylglycerol and phospholipid-activated family of serine-threonine protein kinases. Two classes and at least seven isozymes of PK-C have been described. Class A enzymes are calcium-dependent isozymes while the class B enzymes are calcium-independent. PK-C has been implicated in the regulation of smooth muscle contraction. Nishikawa et al. (1984) have shown that HMM, isolated MLC20 and intact myosin are substrates for PK-C. PK-C phosphorylates serine 1, serine 2 and threonine 9 of the regulatory light chain (Bengar et al., 1987; Ikebe et al., 1987). This phosphorylation increases the $K_M$ of MLCK for serine 19 of the MLC20 (Nishikawa et al., 1984). These data suggest an inhibitory role for PK-C in smooth muscle
contraction. Incubation of skinned vascular smooth muscle with PK-C has been shown to inhibit contraction (Inagaki et al., 1987). This is contradicted by studies correlating PK-C phosphorylation with contraction in smooth muscle. Singer and Baker (1987) have demonstrated that phorbol dibutyrate in the presence of calcium produces contraction in arterial smooth muscle. Smooth muscle contraction was accompanied by an increase in MLC$_{20}$ phosphorylation; however, most of the phosphorylation occurred at a site other than serine 19 (Singer and Baker, 1987; Singer et al., 1989; Kamm et al., 1989). The addition of specific PK-C inhibitors was able to reverse the effects of the PK-C phosphorylation (Sasaki et al., 1990; Singer, 1990a; Brozovich et al., 1990). Recently, PK-C has been shown to induce contraction in aortic smooth muscle; however, in this study serine 19 was the dominant phosphorylated species following exposure to phorbol dibutyrate (Itoh et al., 1993). Phorbol dibutyrate also produced an inhibition of the myosin phosphatase activity. Addition of a PK-C inhibitor restored the phosphatase activity and relaxed the muscle suggesting that PK-C can regulate the myosin phosphatase activity. The different effects of PK-C activation may be due to the different muscle types used in these studies.

2.2.3.2 Thin filament regulation

Thin filaments are composed of actin, tropomyosin, caldesmon and calponin. In skeletal muscle tropomyosin and the troponin complex are involved in the primary regulation of contraction. In contrast, the troponin complex has not been identified in smooth muscle and the role of tropomyosin is uncertain. However, two thin filament proteins, caldesmon and calponin, have been implicated in the regulation of the
contractile process. The following sections review the roles of these proteins in the regulation of muscle contraction.

2.2.3.2.0 Regulation through caldesmon

Caldesmon is a calmodulin-binding thin filament protein shown to interact directly to actin, myosin and tropomyosin. Caldesmon binds to the same N-terminal portion of actin that is involved in the activation of the myosin ATPase activity (Sutoh et al., 1982; Bartegi et al., 1990). Several laboratories have proposed that caldesmon is involved in the regulation of smooth muscle contraction. Caldesmon inhibits the actin-activated Mg$^{2+}$ATPase of myosin (Ngai and Walsh, 1984). Inhibition may be due to the interaction of caldesmon with actin at its myosin binding site (Szpacenko and Dabrowski, 1986) or through the inhibition of product release (Marston, 1988). Marston and Redwood (1992) have shown that the C-terminal 99 amino acids are sufficient to inhibit the actin activation of myosin Mg$^{2+}$ATPase. The inhibitory effect of caldesmon is abolished by Ca$^{2+}$-calmodulin in large molar excess (Hemric and Chalovich, 1988; Velaz et al., 1989).

Caldesmon can be phosphorylated by the multifunctional calcium-calmodulin dependent kinase II in vitro (Ngai and Walsh, 1984; Scott-Woo et al., 1990; Ikebe et al., 1990) on as many as nine residues (Ikebe and Reardon, 1990). Rapid phosphorylation to 1 mole phosphate/mole caldesmon has been shown to reverse the inhibition of the actin-activated myosin-Mg$^{2+}$ATPase (Ngai and Walsh, 1984, 1987). This result is in disagreement with the observation that phosphorylation by calcium-calmodulin dependent kinase II has no effect on the actin-activated myosin-Mg$^{2+}$ATPase (Lash et al., 1986).
Protein kinase C can also phosphorylate caldesmon in vitro (Umekawa and Hikada, 1985; Litchfield and Ball, 1987; Vorotnikov et al., 1988, Tanaka et al., 1990). Phosphorylation has been localized to the C-terminal actin binding region (Vorotnikov et al., 1988) resulting in decreased affinity for both actin and calmodulin, and reversing the inhibition of actin-activated myosin-Mg\textsuperscript{2+}ATPase activity (Tanaka et al., 1990).

Stimulation of smooth muscle cells with KCl, phorbol ester, ouabain, histamine, and endothelin-1 results in the phosphorylation of caldesmon (Adam et al., 1989, 1990). However, phosphopeptides derived from the in vivo phosphorylation show little similarity with those produced in vitro with either PK-C or calcium-calmodulin dependent kinase II (Adam et al., 1989).

Owing to its ability to bind both actin and myosin, it has been suggested that caldesmon may play a role in latch-bridge formation. Caldesmon has been observed to cross-link actin with myosin (Ikebe and Reardon, 1988) consistent with the suggestion that caldesmon binds myosin with its N-terminus and actin with its C-terminus. In fact caldesmon whose C-terminal region was removed by partial proteolysis or through the expression of deletion mutants was unable to bind actin (Szpacenko and Dabrowska, 1986; Redwood et al., 1990). The interaction of caldesmon with the N-terminal myosin binding domain of actin (Bartegi et al., 1990) may account for the decreased rate of ATP hydrolysis observed during the latch state. Contrary to the idea that caldesmon is involved in latch-bridge formation, electron micrograph studies show that caldesmon does not bridge the gap between actin and myosin but binds along the length of the actin fiber (Walsh, 1991).
2.2.3.2.1 Regulation through calponin

The thin filament calmodulin-binding protein calponin has been implicated in a calcium-dependent regulation of smooth muscle tension. Calponin inhibits the actin-activated myosin ATPase (Winder and Walsh, 1990; Abe et al., 1990) by decreasing the $V_{\text{max}}$ (Horiuchi and Chacko, 1991). Inhibition can be reversed by calmodulin but only in the presence of calcium and when a three-fold molar excess of calmodulin is used (Abe et al., 1990). Calponin is a substrate for protein kinase C and calcium calmodulin dependent kinase II in vitro but is not phosphorylated by either cAMP- or cGMP-dependent kinases or by myosin light chain kinase (Winder and Walsh, 1990). Phosphorylation reversed the inhibition of the actomyosin ATPase activity. A chicken gizzard homolog of SMP-I has been identified as the calponin phosphatase (Pato et al., 1993). Barany et al. (1991) have questioned the physiological relevance of these observations since the stimulation of intact smooth muscle with KCl, norepinephrine, histamine, or phorbol ester did not result in the phosphorylation of calponin. However, Pohl et al. (1991) have reported the phosphorylation of calponin in smooth muscle tissue following carbachol stimulation.

Walsh (1991) has proposed a model for the calcium-dependent calponin mediated regulation of smooth muscle tension (Figure 2.5). Under resting conditions, where the free calcium concentration is $10^{-7}$ M and neither calponin nor MLC$_{20}$ are phosphorylated (panel A), calponin is bound to both actin and tropomyosin in the thin filament. An increase in $[\text{Ca}^{2+}]_{i}$ results in the activation of MLCK and calcium calmodulin dependent kinase II and subsequent phosphorylation of MLC$_{20}$ and calponin (panel B). The phosphorylated calponin no longer binds actin and does not interfere with the actomyosin ATPase. At submaximal
calcium concentrations, Walsh proposes that calponin is in a dephosphorylated state while the MLC\textsubscript{20} is phosphorylated owing to the differences in the calcium concentrations required for MLCK and calcium calmodulin dependent kinase II activities. Under these conditions calponin would be in a position to interfere with actomyosin ATPase activity thereby decreasing the rate of cross-bridge cycling and slowing the velocity of shortening (panel C).
Figure 2.5. The role of calponin in the regulation of smooth muscle relaxation. The interaction of myosin and calponin with the thin filament under conditions of resting, contracting and intermediate [Ca$^{2+}$] are shown. This figure was modified from a diagram in Calcium-dependent mechanisms of regulation of smooth muscle contraction by M. Walsh in Biochem. Cell Biol. 69: 771-800, 1991. Used with permission.
3. Materials and Methods

3.0 Materials

3.0.0 Smooth muscle

The smooth muscle used in this study was obtained from turkey gizzards. The gizzards were generously provided by Plains Poultry Ltd. of Wynyard, Saskatchewan. Fresh smooth muscle was used in the preparation of phosphatases while either fresh or frozen smooth muscle was used in the preparation of myosin, heavy meromyosin, and myosin light chains.

3.0.1 Chemicals and proteins

Table 3.1 lists the chemicals used in this study and the names of their suppliers. Table 3.2 lists the names and addresses of the suppliers.

The 20,000 Da myosin light chains and heavy meromyosin used throughout this study were routinely prepared according to the method of Sellers et al. (1981).

In addition to the chemicals listed in Table 3.1 the following reagents were obtained as gifts. Phosphorylase kinase and the catalytic subunit of cAMP dependent protein kinase were provided by Dr. M. Walsh, University of Calgary. Heat stable inhibitor-2, rabbit muscle type-1 protein phosphatase (PP1C'), and phosphorylase a were gifts of Dr. R. Khandelwal, University of Saskatchewan. Recombinant heat stable inhibitor 2 was a gift from Dr. A. DePaoli-Roach, Indiana University. The anti-type-1 monoclonal antibody was a gift from Dr. J. Vandenheede
Katholieke Universiteit, Leuven, Belgium. Tropomyosin and antitropomyosin polyclonal antibodies were provided by Dr. A. Mak, Queens University. The recombinant type-1 phosphatase catalytic subunits $\alpha, \gamma_1, \gamma_2,$ and $\delta$ were provided by Dr. E.Y.C. Lee, University of Miami. Okadaic acid was the gift of Dr. A. Takai, Universitat Heidelberg, Heidelberg, Germany.

Table 3.1. List of chemicals and proteins

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<th>Supplier</th>
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<tr>
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</tr>
<tr>
<td>BAME (Na-benzoyl-L-arginine methyl ester)</td>
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<td>BCIP (5-bromo-4 chloro 3-indoyl phosphate-toluidine salt)</td>
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<td>MOPS (8-(N-Morpholino propanesulphonic acid)</td>
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<td>L-α-phosphatidyl-L-serine</td>
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<td>Ponceau-S</td>
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<td>STI (Soybean trypsin inhibitor)</td>
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### Table 3.2 Names and addresses of suppliers

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<td>Amersham/Searle</td>
<td>Amersham Canada Ltd., Oakville, Ont, Canada</td>
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<tr>
<td>BDH</td>
<td>British Drug House, Saskatoon, Sask, Canada</td>
</tr>
<tr>
<td>Bio-Rad</td>
<td>Bio-Rad Laboratories, Mississauga, Ont, Canada</td>
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<tr>
<td>Boehringer Mannheim</td>
<td>Boehringer Mannheim Canada Ltd., Laval, Quebec, Canada</td>
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<tr>
<td>Calbiochem</td>
<td>Calbiochem Corp., La Jolla, CA, U.S.A.</td>
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<tr>
<td>ICN Biomedicals</td>
<td>ICN Biomedicals Inc., Cleveland, OH, U.S.A.</td>
</tr>
<tr>
<td>Lipidex</td>
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<td>Miles Scientific</td>
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<td>Pharmacia</td>
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<td>Sigma</td>
<td>Sigma Chemical Co., St. Louis, Missouri, U.S.A.</td>
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### Table 3.3 List of buffers

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<tr>
<td>A</td>
<td>0.5 M NaCl, 15 mM Tris-HCl (pH 7.6), 1 mM EGTA, 5 mM EDTA, 1 mM DTT, 0.1 mM PMSF</td>
</tr>
<tr>
<td>B</td>
<td>20 mM KCl, 20 mM Tris-HCl (pH 7.8), 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF</td>
</tr>
<tr>
<td>C</td>
<td>20 mM KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF</td>
</tr>
</tbody>
</table>
**Buffer** | **Composition**
---|---
D | 0.3 M KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF
E | 0.5 M KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 5% glycerol, 1 mM DTT, 0.1 mM PMSF
F | 0.3 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 5% glycerol, 1 mM DTT, 0.1 mM PMSF
G | 20 mM KCl, 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF
H | 50 mM KCl, 50 mM potassium acetate (pH 5.0), 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF
I | 50 mM NaCl, 10 mM MOPS (pH 7.0), 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF
J | 0.5 M NaCl, 15 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 3 mM NaN₃, 1 mM DTT, 0.1 mM PMSF
K | 25 mM NaCl, 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 10 mM MgCl₂, 3 mM NaN₃, 1 mM DTT

3.1 Methods

3.1.0 Enzyme assays

3.1.0.0 Phosphatase activity assay

The phosphatase activity was monitored by the release of $^{32}$P phosphate from the substrate (Pato and Adelstein, 1983). The dephosphorylation was initiated by the addition of the enzyme to a reaction mixture (50 μl) containing the substrate in 50 mM Tris-HCl (pH...
7.4), 1 mM DTT 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. The solution was chilled and centrifuged at 15,000 rpm for 1 minute in a benchtop centrifuge. A 200 μl aliquot of the supernatant was mixed with 4 ml of Formula-963 aqueous scintillation cocktail and counted in a Beckman liquid scintillation counter. The reaction time was determined from the linear portion of the activity curve of the enzyme with respect to reaction time.

The effects of a variety of reagents on SMP-III activity were determined by preincubating the phosphatase with the effector (inhibitor-2, okadaic acid, divalent cations (Ca²⁺, Mg²⁺, Mn²⁺, Cu²⁺, Ni²⁺, Co²⁺), KCl, nucleotides (ATP, GTP, GMP, GDP, cGMP), or NaF) in 50 mM Tris-HCl (pH 7.4) and 1 mM DTT for 5 minutes at room temperature. The reaction was initiated with the addition of substrate (final volume 50 μl) at 30 °C. The reaction was stopped by the addition of TCA. Dephosphorylation was monitored using the method described above.

3.1.0.1 Dephosphorylation of phosphorylase kinase

The time course of dephosphorylation of phosphorylase kinase was determined by taking aliquots of the reaction mixture containing ³²P-labeled phosphorylase kinase (52 μg/ml) in 50 mM Tris-HCl (pH 7.4), 1 mM DTT before and at various time points after the addition of phosphatase. The aliquots were mixed with an equal volume of SDS sample buffer (0.1 M Tris-HCl (pH 6.8), 2% SDS, 20% (v/v) glycerol, bromophenol blue) containing 5% 2-mercaptoethanol and frozen immediately in dry ice. The samples were boiled for 5 minutes and
applied to a 12.5% SDS-polyacrylamide gel. The extent of dephosphorylation was visualized by autoradiography.

3.1.0.2 Kinetic studies

The enzyme assay was performed according to the method described in 3.1.0.0 using either SMP-III prepared as described in section 3.1.2 or the product a 5 minute tryptic digestion of SMP-III produced as described in section 3.1.9. The enzymes were assayed in a mixture containing 50 mM Tris-HCl (pH 7.4), 1 mM DTT and various concentrations of either phosphorylated myosin light chains (1.0-6.7 μM) or phosphorylated heavy meromyosin (1.0 -5.0 μM). The $K_M$ and $V_{max}$ for SMP-III and the 5 minute digest of SMP-III were determined from a double reciprocal Lineweaver Burk plot.

3.1.0.3 Determination of pH profile

The pH profile for SMP-III was determined by assaying the phosphatase in buffers of pH ranging from 5.0 to 9.5. The general composition of the reaction mixture was 100 mM buffer, 1 mM DTT and substrate. The buffers used for the different pH were sodium acetate for pH 5 and 5.5, sodium phosphate for pH 6 and 6.5, MOPS-NaOH for pH 6.5-7.5, Tris-HCl for pH 7.0-8.5, and glycine-HCl for pH 8.5 and 9.

3.1.1 Phosphorylation of substrates

[$\gamma^{32}$P] ATP (1mCi/ml) was used for the phosphorylation of all the substrates.
3.1.1.0 Myosin light chain (MLC$_{20}$), heavy meromyosin (HMM), and myosin phosphorylation

The substrates were prepared from turkey gizzards and were phosphorylated according to Pato and Adelstein (1983). MLC$_{20}$ was incubated with turkey gizzard myosin light chain kinase in 50 mM Tris-HCl (pH 7.4), 0.25 mM CaCl$_2$, 15 mM MgCl$_2$, 0.1 µM calmodulin, and 0.1 mM [$\gamma$-$^{32}$P] ATP (0.1 mCi/ml) for 45 minutes at room temperature. HMM was incubated with turkey gizzard myosin light chain kinase in 50 mM Tris-HCl (pH 7.4), 0.3 mM CaCl$_2$, 4 mM MgCl$_2$, 0.1 µM calmodulin, and 0.3 mM [$\gamma$-$^{32}$P] ATP (0.1 mCi/ml) for 45 minutes. Myosin was incubated with turkey gizzard myosin light chain kinase in 50 mM Tris-HCl (pH 7.4), 1 mM CaCl$_2$, 5 mM MgCl$_2$, 150 mM NaCl, 0.1 µM calmodulin, and 0.3 mM [$\gamma$-$^{32}$P] ATP (0.1 mCi/ml) for 45 minutes. Phosphorylation was determined by spotting 10 µl of reaction mixture onto Whatman p81 paper, washing three times with 75 mM H$_3$PO$_4$ for 5 minutes and once with acetone. The samples were dried and counted in 4 ml of aqueous scintillation fluid in a Beckman LS 7800 liquid scintillation counter. Unreacted ATP was removed by exhaustive dialysis against 20 mM KCl, 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF containing decreasing concentrations (1 M, 0.5 M and 20 mM) of KCl at 4 °C. For substrates used for determining the effect of divalent cations the dialysis buffer did not contain EDTA or EGTA.

3.1.1.1 Phosphorylase kinase phosphorylation

Phosphorylase kinase was phosphorylated according to the method of Ganapathi and Lee (1984). This involves the incubation of rabbit skeletal muscle glycogen phosphorylase kinase in 50 mM imidazole-HCl
(pH 7.2), 7 mM MgCl$_2$, 0.5 mM EGTA, 2 mM EDTA, 1 mM DTT, 50 mM NaF, 0.1 mM [γ-$^{32}$P] ATP (0.1 mCi/ml) and 20 % glycerol at room temperature. The reaction was initiated with the addition of the free catalytic subunit of cAMP-dependent protein kinase and incubated at room temperature for 60 minutes. Aliquots of the phosphorylation mixture were electrophoresed on 12.5% polyacrylamide gels and autoradiographed using Kodak X-ray film to monitor phosphorylation. NaF was removed by centrifugation through a Centricon-10 concentrator with repeated washes with 50 mM Tris-HCl (pH 7.4) and 1 mM DTT. The substrate was stored at -20 °C.

3.1.1.2 Phosphorylation of histone IIA

Histone IIA was phosphorylated with cAMP-dependent protein kinase catalytic subunit. Histone IIA was incubated in 50 mM Tris-HCl (pH 7.4), 10 mM Mg$^{2+}$, and 0.1 mM [γ-$^{32}$P] ATP (0.1 mCi/ml). The reaction was initiated with the addition of the free catalytic subunit of cAMP-dependent protein kinase and incubated at room temperature for 60 minutes. Unreacted ATP was removed by exhaustive dialysis against 20 mM KCl, 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF containing decreasing concentrations (1 M, 0.5 M and 20 mM) of KCl at 4 °C.

3.1.1.3 Phosphorylation of glycogen phosphorylase

Phosphorylated glycogen phosphorylase (phosphorylase a) was a gift from Dr R. Khandelwal, University of Saskatchewan. Phosphorylase a was prepared from phosphorylase b using phosphorylase kinase, [γ-$^{32}$P] ATP and Mg$^{2+}$ according to the method of Krebs et al. (1958).
3.1.2 Purification of SMP-III

3.1.2.0 Phosphatase extraction

Turkey gizzard smooth muscle (300-1000 g) was homogenized in 4 volumes of extraction buffer, 50 mM Tris-HCl (pH 7.6), 10 mM MgAcetate, 15 mM DTT containing protease inhibitors, 0.1 mM PMSF, 10 mg/l STI, 10 mg/l TPCK, 10 mg/l BAME, 1 mg/l leupeptin, and 1 mg/l pepstatin, and stirred for 45 minutes at 4 °C. The homogenate was centrifuged for 15 minutes at 9,000 rpm in a Sorval centrifuge using a GS3 rotor. The supernatant was filtered through cheese cloth and glass wool and fractionated 0-30% saturation then 30-60 % saturation with (NH₄)₂SO₄. The pellet from the 30-60 % fraction was dissolved in a minimum volume of buffer A (0.5 M NaCl, 15 mM Tris-HCl (pH 7.6), 1 mM EGTA, 5 mM EDTA, 1 mM DTT, and 0.1 mM PMSF) and dialyzed against buffer B (20 mM KCl, 20 mM Tris-HCl (pH 7.8), 1 mM EGTA, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF).

3.1.2.1 Column chromatography

The dialysed phosphatase solution was centrifuged and applied to a DEAE-Sephacel column (5x8 cm) by peristaltic pump (100 ml/hr). The column was washed with buffer B and eluted with a 20-500 mM KCl linear gradient. Fractions with phosphatase activity were pooled and precipitated with 60 % saturated (NH₄)₂SO₄. Following centrifugation the pellet was dissolved in a minimum volume of buffer A and dialysed overnight against the same buffer.

The phosphatase solution was centrifuged and applied to a Sephacryl S-300 column (5x87 cm) by pump (100 ml/hr). Phosphatase was
eluted with buffer A. This step separates the phosphatase activity into three peaks. The first peak contains SMP-III.

The impure SMP-III was chromatographed on a Heparin-Sepharose (1.5x8 cm) column. The column was washed with buffer C (20 mM KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF) and eluted with a 20-800 mM linear gradient at ~60 ml/hr. Fractions containing phosphatase activity were pooled and dialysed against buffer C to remove salt.

Partially purified SMP-III was further chromatographed on a thiophosphorylated HMM Sepharose affinity column (2.5x6 cm). The column was washed with buffer C and eluted with the same buffer containing 0.8 M KCl. Fractions containing phosphatase activity were pooled and dialysed against buffer C containing 5 mM MgCl₂.

SMP-III was reapplied to the affinity column that had been equilibrated with buffer C containing 5 mM MgCl₂. The column was eluted with buffer C containing 0.8 M KCl. Fractions containing phosphatase activity were pooled and dialysed against buffer C.

Purified SMP-III was concentrated by chromatography on an AH Sepharose column (1x0.5 cm). Fractions containing phosphatase activity were pooled and dialysed against 100 ml of buffer C containing 50% glycerol. SMP-III was stored in -20 °C. When stored in this manner, SMP-III is stable for at least 6 months.

3.1.3 Purification of myofibril associated phosphatase

Purification of this phosphatase was carried out using the same procedure as described for SMP-III with the noted exceptions.
3.1.3.0 Phosphatase extraction

The myofibril pellet obtained after the centrifugation of the low salt extraction described above was re-extracted with the same extraction buffer containing 0.5 M KCl. The supernatant was fractionated with (NH₄)₂SO₄ and the 0-60% fraction was dialysed against buffer A overnight. The dialysate was centrifuged and the supernatant, which contains the phosphatase activity, was further purified.

3.1.3.1 Column chromatography

The myofibril phosphatase was chromatographed on the DEAE-Sephalcel, Sephacryl S-300, and Heparin-Sepharose columns as described in section 3.1.2.1. The final chromatography step was carried out on a thiophosphorylated myosin light chain Sepharose affinity column. The column was washed stepwise first with 20 mM KCl then 0.13 M KCl in buffer C and then eluted with 1.0 M KCl in buffer C.

The myofibril phosphatase was concentrated by chromatography on AH sepharose. Fractions containing phosphatase activity were pooled and dialysed against 100 ml of buffer C containing 50% glycerol. The myofibril-associated phosphatase was stored at -20°C.

3.1.4 Fast protein liquid chromatography (FPLC)

Fast protein liquid chromatography was used during the first attempts to purify smooth muscle phosphatase-III. The gel filtration column Superose 12 HR 10/30, the cation exchange column Mono S HR5/5, and the anion exchange column Mono Q HR 5/5 were used in the purification of SMP-III. Superose 12 HR 10/30 was also used to determine the effects of GTP on the subunit composition of SMP-III.
Superose 12 HR10/30 is a gel filtration column with an efficient separation range of 1,000 to 300,000 Da. Crude phosphatase preparations were chromatographed using several buffer compositions. Buffer D contained 0.3 M KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF. Buffer E contained 0.5 M KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 5% glycerol, 1 mM DTT, and 0.1 mM PMSF. Buffer F contained 0.3 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 5% glycerol, 1 mM DTT, and 0.1 mM PMSF. Flow rates from 0.4 to 0.8 ml/minutes were used.

Mono Q HR5/5 is an anion exchange column utilizing the charged group \(-\text{CH}_2\text{N}^+\text{(CH}_3)_3\). Crude myosin phosphatase was chromatographed at 1 ml/minute using a buffer G containing 20 mM KCl, 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF and eluted with a 20-1000 mM KCl gradient in the same buffer.

Mono S HR5/5 is a cation exchange column utilizing the charged group \(-\text{CH}_2\text{SO}_3^-\). Crude phosphatase was chromatographed at 1 ml/minute using a buffer H containing 50 mM potassium acetate (pH 5.0), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF and eluted with a gradient of 50-1000 mM KCl in the same buffer. To neutralize the eluate, a 15 μl aliquot of 1.0 M Tris-HCl pH 7.4 was added in advance to each tube.

3.1.5 Molecular weight determination by gel filtration on Sephadex G-200

A Sephadex G-200 gel filtration column (0.9x60 cm) was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa). Proteins were chromatographed at 8.8 ml/hour with 0.5 M NaCl, 20 mM Tris-HCl (pH 7.6), 0.1 mM EGTA, 0.5 mM EDTA,
and 1.0 mM DTT. A standard curve was produced by plotting the elution volume of each protein against the log of their molecular weights. The molecular weight for SMP-III was determined from this standard curve.

3.1.6 Preparation of the catalytic subunit of SMP-III (SMP-IIIc)

The catalytic subunit of SMP-III was prepared by ethanol precipitation according to the method of Brandt et al. (1975). SMP-III was precipitated with 4 volumes of ice cold 95 % ethanol. The solution was centrifuged for 1 minute at 15,000 rpm in a benchtop centrifuge. The supernatant was removed and the pellet was extracted with buffer C. The solution was centrifuged immediately. The supernatant was removed, dialyzed against buffer C and assayed for phosphatase activity. SMP-IIIc was concentrated by dialyzing against buffer C containing 50 % glycerol and stored at -20 °C.

3.1.7 Preparation of the affinity column

3.1.7.0 Coupling of the protein to the resin

Heavy meromyosin Sepharose was prepared by coupling HMM to CNBr-activated Sepharose 4B according to the manufacturer's directions. Heavy meromyosin (145 mg) was dissolved in 15 ml coupling buffer (0.1 M NaHCO₃ (pH 8.3), 0.5 M NaCl) and dialyzed against the same buffer overnight. CNBr-activated Sepharose 4B (10 g) was swollen in 60 ml of 0.1 mM HCl for 20 minutes at room temperature. The resin was washed with 1 l of 1 mM HCl followed by 200 ml of coupling buffer in a sintered glass filter. The resin was suspended in 20 ml of coupling buffer. The HMM solution was added to the resin and the mixture was mixed gently for 10 hours at 4 °C. To determine the extent of coupling an aliquot of the
mixture was centrifuged at 15,000 rpm in a benchtop centrifuge and the protein concentration of the supernatant was determined using Bio-Rad protein assay reagent. When no more protein was found in the supernatant, the unreacted active groups of the resin were blocked by washing with 0.1 M Tris-HCl (pH 8.0).

3.1.7.1 Thiophosphorylation of heavy meromyosin-Sepharose

The HMM-Sepharose was washed with buffer containing 0.5 M KCl, 50 mM Tris-HCl (pH 7.4), 0.3 mM CaCl₂, 10 mM MgCl₂ and 4 mM DTT. The resin was suspended in 20 ml of the same buffer. ATPγ-S and calmodulin were added to a final concentration of 0.7 mM and 0.1 μM, respectively. Phosphorylation was initiated by the addition of turkey gizzard myosin light chain kinase. The mixture was incubated with rocking at room temperature for 8 hours and then overnight at 4 °C. The resin was washed with buffer C containing 0.5 M KCl to prepare the resin for use.

3.1.8 Electrophoresis

3.1.8.0 SDS-polyacrylamide microslab gel electrophoresis

Slab gel electrophoresis was performed according to the method of Laemmli (1970) using 12.5% polyacrylamide gels (0.75 mm thick) with 5% stacking gel. The gel had a final composition of 12.5% acrylamide, 0.375 M Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.05% (v/v) TEMED, and 0.05% (w/v) ammonium persulfate. The composition of the stacking gel was 5% acrylamide, 0.125 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.05% (v/v) TEMED, and 0.05% (w/v) ammonium persulfate. The electrode buffer was 25 mM Tris (pH 8.2), 192 mM glycine and 0.1% (w/v) SDS.
The samples were diluted 1:1 in SDS PAGE sample buffer (0.1 M Tris-HCl, pH 6.8, 2% SDS (w/v), 20% glycerol (v/v), bromophenol blue) containing 5% (v/v) 2-mercaptoethanol and boiled for 5 minutes. The samples were applied to the gel and electrophoresed at 175 volts for 1.5 hours. The gel was stained for 5 minutes in 0.25% Coomassie Blue in methanol and acetic acid and destained with a 10% methanol and 7.5% acetic acid solution. The gel was soaked in a 10% glycerol solution and air dried at room temperature between two sheets of cellophane saturated with 10% glycerol.

3.1.8.1 Urea, SDS-polyacrylamide microslab gel electrophoresis

These gels were made much like the SDS-polyacrylamide gels described above. 12.5% acrylamide gels (0.75 mm thick) with a 5% stacking gel containing 2M urea with a ratio of 30% acrylamide to 0.8% N,N’-bis acrylamide. The gel had a final composition of 12.5% acrylamide, 0.375 M Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 2 M urea, 0.05% (v/v) TEMED, and 0.05% (w/v) ammonium persulfate. The composition of the stacking gel was 5% acrylamide, 0.125 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 2 M urea, 0.05% (v/v) TEMED, and 0.05% (w/v) ammonium persulfate. The electrode buffer was 25 mM Tris (pH 8.2), 192 mM glycine and 0.1% (w/v) SDS.

The samples were diluted 1:1 in SDS PAGE sample buffer containing 5% (v/v) 2-mercaptoethanol and 2 M urea and boiled for 5 minutes. The samples were applied to the gel and electrophoresed at 175 volts for 1.5 hours. The gel was either stained with Coomassie Blue as described above or transblotted to Immobilon S or nitrocellulose as described in sections 3.1.9. and 3.1.9.0.
3.1.8.2 Non-denaturing polyacrylamide tube gel

Non-denaturing tube gel electrophoresis was performed using 3.5% gels by a modified procedure described by Fairbanks et al. (1971). The gel had a final composition of 0.4 M Tris-Acetate (pH 7.4), 0.2 M NaAcetate, 20 mM EDTA, 0.05% (v/v) TEMED, and 0.05% (w/v) ammonium persulfate. The running buffer was 0.4 M Tris-Acetate (pH 7.4), 0.2 M NaAcetate, 20 mM EDTA. The gel was pre-run overnight at 2 mA per tube. The sample in 50% glycerol was applied to the gel and run at 2 mA per tube for 30 minutes and then 2.5 mA per tube for 10 hours. The gel was carefully removed from the tube and cut into 2 mm slices. Each slice was incubated in 100 μl elution buffer (50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 0.2 mg/ml β-lactoglobulin, and 1 mM DTT) overnight at 4 °C with gentle shaking. The eluates were assayed for activity against phosphorylated MLC_{20} and HMM.

3.1.9 Western blot analysis

Transblot of the proteins from a microslab gel (SDS PAGE, Urea-SDS PAGE and non-denaturing PAGE) to a nitrocellulose paper was carried out according to the procedure of Towbin et al. (1979). The gel was soaked in transfer buffer (192 mM glycine, 25 mM Tris, and 20% methanol) and the transblot was carried out in the same buffer at 35 mV for 3 hours at room temperature. After the electrotransfer, the nitrocellulose sheet was stained briefly with amido black (0.1% amido black in 45% methanol and 10% acetic acid) to visualize and mark the position of the molecular weight standards. The nitrocellulose paper was destained by washing with water. The blot was then washed with TBS (20 mM Tris-HCl (pH 7.5), 0.5 M NaCl) and incubated with 3% gelatin in TBS
for 1 hour to block the unreacted groups of the nitrocellulose. The paper was washed with TTBS (0.05 % (v/v) TWEEN-20, 20 mM Tris-HCl (pH 7.5), 500 mM NaCl) then incubated with the appropriate anti-phosphatase antibodies in 1% gelatin and TTBS overnight. Finally, the blot was washed in TTBS and incubated with the appropriate secondary antibodies conjugated with alkaline phosphatase. The cross-reactivity was visualized using the color development reagents BCIP (30 mg/ml) and NBT (15 mg/ml). The reagents were dissolved in 70% DMS and then diluted in 0.1M NaHCO₃-NaOH (pH 9.8) and 1 mM MgCl₂ to a final concentration of 0.3 and 0.15 mg/ml, respectively.

3.1.9.0 Transblot onto Immobilon-P

Proteins were transferred from a urea-SDS polyacrylamide gel to Millipore Immobilon-P in a similar manner to the transfer described under western blot analysis. The gel was soaked in transfer buffer and the transblot was carried out at 35 mV for 3 hours. The proteins were visualized with 0.1% Ponceau-S in 1% acetic acid. The 38 kDa protein band was cut out, dried, and stored at -20 °C.

3.1.10 Trypsin digestion

L-tosyl-amido-2-phenylethylchloromethyl ketone treated trypsin (50 μg/ml) was added to the phosphatase in 50 mM Tris-HCl (pH 7.4), 1 mM DTT, 1 mg/ml bovine serum albumin to a final concentration of 5 μg/ml at room temperature. Aliquots were taken before and at various times after the addition of trypsin. Tryptic digestion was terminated by pipetting the aliquots into tubes containing soybean trypsin inhibitor, 10-fold the concentration of trypsin in the aliquot. The digests were kept in ice until
they were assayed. Digests used for western blot analysis were frozen immediately in dry ice, boiled for 5 minutes to denature the enzymes, and dried in a Savant Speed Vac Concentrator. The pellet was suspended in SDS PAGE sample buffer containing 2-mercaptoethanol and stored at -20 °C.

3.1.11 Preparation of myosin

Turkey gizzard myosin was prepared according to the procedure of Sellers and Pato (1984). Gizzard muscle was minced in a Waring blender and homogenized in 4 volumes of buffer I (50 mM NaCl, 10 mM MOPS (pH 7.0), 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT, and 0.1 mM PMSF) with 0.5% Triton X-100. The homogenate was centrifuged for 15 minutes at 7,000 rpm in a Sorval RC5 centrifuge using a Sorval GS3 rotor. The pellet was washed 4 times in buffer I to remove the Triton X-100 and homogenized in 40 mM NaCl, 40 mM MOPS (pH 7.2), 1 mM EDTA, 2 mM EGTA, 10 mM ATP. Following a 20 minute centrifugation at 11,000 rpm (Sorval GSA rotor) the supernatant was made to 0.6 M NaCl, 20 mM MgSO₄ and 15 mM ATP and precipitated with 42% saturated (NH₄)₂SO₄. The solution was stirred at 4 °C and centrifuged for 30 minutes at 11,000 rpm. The supernatant was removed, made to 53% saturated NH₂(SO₄)₂, stirred at 4 °C and centrifuged. The resulting pellet was dissolved in a minimum volume of 25 mM NaCl, 5 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM MgCl₂, 1 mM DTT, and 0.1 mM PMSF and dialysed overnight against the same buffer. The solution was diluted with an equal volume of H₂O and made to 10 mM MgCl₂. Following centrifugation the pellet was washed with buffer A and homogenized with a glass homogenizer in buffer I containing 3 mM NaN₃. 10 ml of 6 mg/ml crude myosin in buffer
J (0.5 M NaCl, 15 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 3 mM NaN₃, 1 mM DTT, and 0.1 mM PMSF) containing 0.1 M ATP and 1.0 M MgCl₂ was chromatographed on a Sepharose 4B gel filtration column and eluted with buffer J. Myosin containing fractions were found by applying aliquots to 12.5% SDS polyacrylamide gels and visualizing with Coomassie Blue. The myosin was precipitated by making solution to 10 mM MgCl₂. Following centrifugation the myosin pellet was suspended in buffer K (25 mM NaCl, 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 10 mM MgCl₂, 3 mM NaN₃, and 1 mM DTT), homogenized in a glass homogenizer, and stored at 4 °C.

3.1.12 Myosin binding studies

The extent of phosphatase binding to myosin was determined using the method of Sellers and Pato (1984). Phosphatase was incubated with various concentrations of myosin in binding buffer (50 mM NaCl, 10 mM MOPS, 1 mM MgCl₂) for 5 minutes and centrifuged in a Beckman Airfuge at 30 psi for 15 minutes. Samples were kept on ice until assayed. The extent of phosphatase binding was determined from the relative phosphatase activity remaining in the supernatant.

The effect of a variety of compounds on myosin binding affinity was determined by incubating the effector with the phosphatase and myosin prior to centrifugation. The relative effects were determined from the comparison of phosphatase activity in the supernatant activity with and without effector.

3.1.13 Chemical cross-linking

Phosphatase catalytic subunit of SMP-III was chemically cross-linked to associated proteins using chemical cross-linking reagent DSP
[dithiobis(succinimidyIpropionate)] according to the manufacturer's instructions. SMP-III was incubated in 20 mM sodium phosphate buffer (pH 7.4), 0.15 M NaCl with 0.05 or 0.1 mM DSP for 30 minutes at room temperature. The reaction was quenched with the addition of Tris-HCl (pH 7.4) to a final concentration of 25 mM. The cross-link was cleaved with 5% (v/v) 2-mercaptoethanol and boiling for 5 minutes.

3.1.14 GTP photoaffinity labeling

Smooth muscle proteins were photoaffinity labeled with GTP according to the procedure of Im and Graham (1990). Protein was incubated in 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 0.1 M NaCl, 0.5 mM DTT, and 0.5 mM App(N)p (adenyl-5'-yl imidodiphosphate) for 5 minutes at room temperature. [α-32P] GTP (specific activity 2 mCi/ml) was added to a final concentration of 0.2, 2.0, or 20 μM and allowed to incubate for 45 minutes at room temperature. The samples were placed on ice and cross-linking was initiated with short wavelength UV radiation with the light source placed 4 cm from the samples. The reaction was stopped with the addition of an equal volume of SDS PAGE sample buffer. The samples were boiled for 5 minutes and stored at -20 °C.

3.1.15 Immunoprecipitation

Crude SMP-III (150 μl) was incubated with anti-type-1 phosphatase monoclonal antibody (10 μl) on ice with rocking. Pansorbin (45 μl) was added and the mixture was incubated on ice for 1 hour. The mixture was centrifuged for 2 minutes at 15,000 rpm in a benchtop centrifuge. The supernatant was removed and dried in a Savant Speed Vac Concentrator. The pellet was washed twice in buffer (15 mM Tris-HCl (pH 7.6), 0.5 M
NaCl, 1 mM EDTA, 5 mM EGTA) containing 0.1% (v/v) Nonidet P-40 and once in buffer without Nonidet P-40. The pellet was dissolved in 0.1 M Tris-HCl (pH 6.8), 2% SDS (w/v), 20% glycerol (v/v), bromophenol blue, and 5% (v/v) 2-mercaptoethanol, boiled for five minutes and applied to a 12.5% SDS-polyacrylamide gel. Proteins were visualized by Coomassie Blue staining, autoradiography, or western blot analysis.

3.1.16 Determination of protein concentration

Protein concentration was determined by the method of Bradford (1976) using a Bio-Rad protein assay kit. This method is based on a shift in maximum absorbance of an acidic Coomassie brilliant blue G-250 from 465 to 595 nm upon interaction with protein. The microassay procedure was performed according to the manufacturers instructions. Bio-Rad dye reagent concentrate was diluted 1 into 5 with distilled, deionized water. A standard curve was obtained by determining the absorbance at 595 nm of 0.5-4.0 μg of bovine serum albumin in 1 ml of dye reagent. The protein concentration of an unknown solution was determined from the absorbance at 595 nm of a known volume of that solution.

3.1.17 Phosphorylation of SMP-III preparation with PK-C

Lipid micelles were prepared from solutions of diolein (10 mg/ml) and phosphatidylserine (2.5 mg/ml) in chloroform. 50 μl of phosphatidylserine and 2.5 μl of diolein were combined in a 1.5 ml eppendorf tube and dried under N₂ gas. 250 μl of 50 mM Tris-HCl (pH 7.4) was added and the mixture was sonicated for 2 minutes to form micelles. SMP-III was incubated in buffer containing 125 mM Tris-HCl (pH 7.4), 0.5 mM CaCl₂, 25 mM MgCl₂, 0.25 μM calmodulin, 125 μg/ml
phosphatidylserine, 25 μg/ml diolein, 0.3 mM [γ-32P] ATP. The reaction was initiated with the addition of protein kinase C and incubated at room temperature for 45 minutes. The phosphorylated enzyme was kept on ice until assayed. The effect of phosphorylation on enzyme activity, sensitivity to heat stable inhibitor-2, and myosin binding properties were determined according to the methods described in section 3.1.0.0 and 3.1.12. The extent of phosphorylation was determined by autoradiography.
4.0 Results

The contraction and relaxation of smooth muscle depends on the reversible phosphorylation of MLC\textsubscript{20}. The phosphorylation reaction, catalyzed by MLCK, has been well characterized. However, little is known about the dephosphorylation reaction. In order to study this event we set out to purify and characterize the myosin phosphatase involved in the dephosphorylation of MLC\textsubscript{20}. Turkey gizzards were chosen as the source for smooth muscle because they are readily available, inexpensive, and the smooth muscle component is easily separated from the other tissue components. Moreover, numerous studies on the biochemical properties of other contractile proteins such as myosin, MLCK, caldesmon and calponin and physiological studies of the contractile apparatus of the turkey gizzard smooth muscle have been carried out.

Previous studies using turkey gizzard smooth muscle have shown the existence of four distinct protein phosphatases in the cytosolic fraction. Three of these enzymes, smooth muscle phosphatases (SMP)-I, -II, and -IV have been purified to homogeneity. The fourth enzyme, a myosin phosphatase termed SMP-III, is the main focus of this study. More recently, phosphatase activity has been found in the myofibril fraction. We, subsequently, purified a myosin phosphatase from this fraction and comparisons were made with SMP-III.
4.1 Purification of SMP-III

The first step in this project was the purification of SMP-III. Fresh turkey gizzards were essential for the purification since previous studies have shown extensive degradation of phosphatases isolated from frozen tissue. The purification procedure involved the fractionation of the cytosolic extract with (NH₄)₂SO₄ and chromatography on ion exchange, gel filtration and affinity columns. The column fractions obtained were assayed routinely for phosphatase activity towards both MLC₂₀ and HMM, and the phosphatase-containing fractions were examined by SDS PAGE to determine the level of purity achieved.

4.1.0 Extraction

The first steps of the purification of SMP-III were modeled after the purification of the other smooth muscle phosphatases. Purification was initiated with the homogenization of turkey gizzard smooth muscle and the subsequent separation of the cytosolic component from the myofibril component by centrifugation. This was followed by fractionation with ammonium sulfate. About 90% of the phosphatase activity was found in the 30-60% saturated ammonium sulfate fraction (Pato and Adelstein, 1983).

4.1.1 DEAE-Sephacel chromatography

Column chromatography was used to further purify SMP-III. All chromatography was carried out at 4 °C unless otherwise stated. Significant purification was achieved by chromatography on DEAE-Sephacel as indicated in figure 4.1 which shows the elution profile for this column. All of the protein phosphatase activity bound to the resin and a
single phosphatase peak was eluted from the column at 250-400 mM KCl. The absorbance profile shows the separation of the phosphatase activity from a large proportion of contaminating proteins.

Figure 4.1. Elution profile for the chromatography of SMP-III on DEAE-Sephacel. The column (5x8 cm) was run at 100 ml/hr, eluted with a linear 20-500 mM KCl gradient and 12.5 ml fractions were collected. The fractions were assayed for phosphatase activity towards MLC_{20} (□) and HMM (●). The absorbance at 280 nm is shown (-).

4.1.2 Sephacryl S-300 gel filtration chromatography

It has been previously reported that the smooth muscle phosphatases can be fractionated by molecular sieve chromatography (Pato and Adelstein, 1983). For this reason the impure phosphatase preparation obtained from DEAE-Sephacel was chromatographed on a Sephacryl S-300
gel filtration column. The fractions were assayed for activity towards MLC_{20} and HMM. The assay mixture for MLC_{20} contained 10 mM Mg^{2+} in order to detect the SMP-II (PP2C) activity. Figure 4.2 shows the fractionation of impure phosphatases into three distinct peaks of phosphatase activity. The first peak (A) contains SMP-III which is active towards both MLC_{20} and HMM (specific activities shown in table 4.1). The apparent absence of activity towards MLC_{20} in peak A (figure 4.2) may be

Figure 4.2. Elution profile for the chromatography of SMP-III on Sephacryl S-300. The column (5x87 cm) was equilibrated at 100 ml/hr and 8.5 ml fractions were collected. The fractions were assayed for phosphatase activity towards MLC_{20} (□) and HMM (●). The absorbance at 280 nm is shown (–).
due to the presence of Mg$^{2+}$ in the assay mixture which inhibits SMP-III activity towards MLC$_{20}$. Peak B, which is also active towards MLC$_{20}$ and HMM, contains both SMP-I and SMP-IV. Peak C contains SMP-II which is active only towards MLC$_{20}$. Purification of SMP-III was continued using peak A.

4.1.3 Heparin-Sepharose chromatography

It has been reported that some phosphatases bind to Heparin-Sepharose. This property was utilized to further purify SMP-III. Impure SMP-III eluted from Sephacryl S-300 was chromatographed on Heparin-Sepharose. The elution profile is shown in figure 4.3. SMP-III bound strongly to the resin while a large portion of the impurities did not. SMP-III was eluted from the resin at 0.4-0.5 M KCl while many contaminating proteins eluted from the column at lower ionic strength. Analysis of the SMP-III peak on SDS PAGE showed the presence of many bands.

4.1.4 Fast protein liquid chromatography (FPLC)

The partially purified SMP-III following chromatography on DEAE-Sephacel, Sephacryl S-300 and Heparin-Sepharose showed numerous bands on SDS PAGE. Attempts were made to further purify the enzyme by FPLC. FPLC was carried out at room temperature. Elution buffers were kept on ice as were the tubes into which the fractions were collected.
Figure 4.3. Elution profile for the chromatography of SMP-III on Heparin-Sepharose. The column (1.5x5 cm) was run at 60 ml/hr, eluted with a linear 20-800 mM KCl gradient, and 2.4 ml fractions were collected. The fractions were assayed for phosphatase activity towards MLC_{20} (□) and HMM (●). The absorbance at 280 nm is shown (-).

4.1.4.0 Superose 12

Impure SMP-III from Heparin-Sepharose was subjected to gel filtration on a Superose 12 HR10/30 column using buffer D (0.3 M KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF) and buffer E (0.5 M KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 5 % glycerol, 1 mM DTT, and 0.1 mM PMSF). Chromatography was first attempted with
buffer D but the phosphatase activity was unstable following elution. Buffer E, used in subsequent chromatography on Superose 12, included glycerol to increase phosphatase stability. Figure 4.4 shows the elution profile of the chromatography of SMP-III on Superose HR10/30 using buffer E. One peak of phosphatase activity was obtained but analysis of the phosphatase-containing fractions by SDS PAGE indicated that little further purification was achieved with this step. Also, eluates from Superose 12 HR10/30 were typically very dilute and readily lost activity.

Figure 4.4. Elution profile for the chromatography of SMP-III on Superose 12 HR10/30. The column (1x30 cm) was run at 0.8 ml/min using buffer F and 0.5 ml fractions were collected. The fractions were assayed for phosphatase activity towards MLC$_{20}$ (square) and HMM (circle).
4.1.4.1. Mono Q HR5/5

The ion exchange resin Mono Q was used in an attempt to further purify SMP-III. Chromatography on Mono Q resulted in the elution of a single phosphatase peak (figure 4.5). Examination of the phosphatase containing fractions on SDS PAGE showed that no significant purification was achieved.

![Graph showing elution profile](image)

Figure 4.5. Elution profile for the chromatography of SMP-III on Mono Q HR5/5. The column (0.5x5 cm) was run at 1.0 ml/min and 0.5 ml fractions were collected. The fractions were assayed for phosphatase activity towards MLC\textsubscript{20} (□) and HMM (●).
4.1.4.2 Mono S HR5/5

The chromatography of SMP-III on Mono S was carried out at pH 5.0. The eluate was collected into tubes containing Tris-HCl pH 7.4 in order to restore a neutral pH. Unlike the chromatography on the other FPLC columns, several peaks of phosphatase activity were obtained from Mono S (figure 4.6). But since chromatography at low pH resulted in significant loss of phosphatase activity Mono S was not used for the purification of SMP-III.

![Elution profile graph](image)

Figure 4.6. Elution profile for the chromatography of SMP-III on Mono S HR5/5. The column (0.5x5 cm) was run at 1 ml/min and 0.5 ml fractions were collected. The fractions were assayed for phosphatase activity towards MLC20 (□) and HMM (●).
4.1.5. Poly-L-lysine agarose

Protein phosphatases have been reported to bind to poly-L-lysine. Thus, impure SMP-III was chromatographed on a poly-L-lysine agarose column in an attempt to further purify the phosphatase. The elution profile shows that most of the protein bound to the column (figure 4.7). A single protein peak that contained phosphatase activity towards both MLC\textsubscript{20} and HMM was eluted at 450-570 mM KCl. Analysis of the eluted sample on SDS PAGE showed little improvement in the purity of SMP-III. Therefore, chromatography on poly-L-lysine was not included in the purification scheme.

4.1.6 Hydrophobic column chromatography

In the search for a resin to further purify SMP-III, its binding to hydrophobic resins (agarose-C\textsubscript{n} series) was examined. Impure SMP-III was applied to several hydrophobic columns with side chains of n= 0, 2, 4, 6, or 8 carbons. The columns were washed in a KCl free buffer and then eluted stepwise with KCl in concentrations ranging from 0.1 to 1.0 mM. The phosphatase activity and the protein concentration of the flow through and eluate of each column were determined. A single protein peak which contained the phosphatase activity was found in all cases. Phosphatase activity did not bind to resins with side chains of n= 0, 2 or 4. Phosphatase activity was eluted from the resin n= 6 with 0.1-0.3 M KCl and from resin n= 8 with 0.1-0.5 M KCl. These results showed that the hydrophobic resins were not appropriate for the purification of SMP-III.
Figure 4.7. Elution profile for the chromatography of SMP-III on poly-L-lysine. The column (1.5x3 cm) was run at 82 ml/hr, eluted with a 20-600 mM KCl linear gradient and fractions of 2.6 ml were collected. The fractions were assayed for phosphatase activity towards MLC$_{20}$ (□) and HMM (●). The absorbance at 280 nm is shown (−).

4.1.7 Affinity chromatography

Affinity chromatography is a powerful technique which has been used successfully in the purification of many proteins. Thus, a number of different affinity columns were tested during the purification of SMP-III. The ligands for these resins including thiophosphorylated MLC$_{20}$-Sepharose, HMM-Sepharose and thiophosphorylated HMM-Sepharose were based on the substrates of SMP-III.
4.1.7.0 Thiophosphorylated MLC$_{20}$-Sepharose Chromatography

Thiophosphorylated MLC$_{20}$-Sepharose was the first affinity resin to be tested. Figure 4.8 shows the elution profile for thiophosphorylated MLC$_{20}$-Sepharose. The profile indicates that the most of the protein bound to the resin and was eluted with 1.0 M KCl. The phosphatase activity was found in the high salt eluate. Since only a small degree of

![Graph showing elution profile](image)

Figure 4.8. Elution profile for the chromatography of SMP-III on thiophosphorylated MLC$_{20}$-Sepharose. Following sample application, the column (1.5x10.5 cm) was washed with buffer C (20 mM KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF) and eluted with buffer C containing 1 M KCl at 37.5 ml/hr. Fractions of 5 ml were collected in the flow through while the fraction volume was decreased to 1 ml for the eluate. The fractions were assayed for phosphatase activity towards HMM (●). The absorbance at 280 nm is shown (●).
purification was obtained by chromatography on thiophosphorylated MLC$_{20}$-Sepharose this resin was not used for the purification of SMP-III.

4.1.7.1 HMM-Sepharose chromatography

Impure SMP-III eluted from Heparin-Sepharose was chromatographed on HMM-Sepharose. The elution profile (figure 4.9) shows none of the protein bound to the resin. All of the phosphatase activity was found in the flow through.

![Graph showing elution profile](image)

Figure 4.9. Elution profile for the chromatography of SMP-III on HMM-Sepharose. The column (1x5 cm) was washed with buffer C and eluted with a linear 20-1000 mM KCl gradient at 54 ml/hr. The fractions (1 ml) were assayed for phosphatase activity towards MLC$_{20}$ (□) and HMM (●). Absorbance at 280 nm is shown (—).
4.1.7.2 Thiophosphorylated HMM-Sepharose chromatography

Partially purified SMP-III eluted from Heparin-Sepharose was chromatographed on thiophosphorylated HMM-Sepharose. Figure 4.10 shows that most of the protein did not bind to the column but all of the phosphatase activity was present in the 0.8 M KCl eluate. A significant increase in purity was achieved in this step. A linear gradient has also been used for the elution of this phosphatase, however, this procedure resulted in a broader and more dilute peak of phosphatase activity with no improvement in purification.

![Graph showing elution profile](image)

Figure 4.10. Elution profile for the chromatography of SMP-III on thiophosphorylated HMM-Sepharose. The column (2.5x6 cm) was run at 54 ml/hr and 0.75 ml fractions were collected. The fractions were assayed for phosphatase activity towards MLC$_{20}$ (□) and HMM (●). The absorbance at 280 nm is shown (○).
A further and dramatic increase in SMP-III purity was achieved by re-chromatographing the partially-purified eluate obtained from thiophosphorylated HMM-Sepharose on the same column in the presence of 5 mM Mg$^{2+}$ (figure 4.11). The elution of the contaminants in the flow through suggests that Mg$^{2+}$ may have altered the properties of the contaminating proteins such that they no longer bind to thiophosphorylated HMM. All of the phosphatase activity bound to the resin and was eluted with 0.8 M KCl.

Figure 4.11. Elution profile for the chromatography of SMP-III on thiophosphorylated HMM-Sepharose in the presence of 5 mM Mg$^{2+}$. The column (2.5x6 cm) was run at 54 ml/hr and 0.7 ml fractions were collected. The fractions were assayed for phosphatase activity towards MLC$_{20}$ (□) and HMM (●). The absorbance at 280 nm is shown (-).
4.1.8 Summary of purification

A summary of the purification scheme for SMP-III is shown in figure 4.12. Turkey gizzard smooth muscle was homogenized in extraction buffer, centrifuged and the supernatant was filtered through cheese cloth and glass wool. The cytosolic component was fractionated with ammonium sulfate and the 30-60 % saturated fraction was chromatographed on DEAE-Sephacel. The phosphatase containing fractions were pooled, concentrated and chromatographed on a Sephacryl S-300 gel filtration column. This step separated the different phosphatases from each other. Purification was continued with peak A which contains SMP-III. Heparin-Sepharose and affinity column chromatography were used to further purify SMP-III. SMP-III was concentrated by chromatography on AH-Sepharose and dialysis against buffer C containing 50 % glycerol. SMP-III is stored at -20 °C and is stable for at least 6 months. SDS PAGE analysis of the purified phosphatase shows three major protein components at 22, 38, and 130 kDa (figure 4.13).

The purification of SMP-III resulted in a 2500-and 888-fold purification of HMM and MLC$_{20}$ phosphatase activities, respectively, from turkey gizzard smooth muscle extract (table 1). The difference in fold purification for the two substrates is due to the presence of four protein phosphatases in the smooth muscle cytosolic extract. Two of these phosphatases, SMP-I and II, are active only towards MLC$_{20}$ and are separated from SMP-III by gel filtration on Sephacryl S-300. Purified SMP-III has specific activities of 444 and 501 nmol/min/mg for MLC$_{20}$ and HMM, respectively. The yield was low with only 30 μg of SMP-III from 360
g of smooth muscle. The purification of SMP-III was carried out 18 times over the course of this project.

![Diagram of purification process]

Figure 4.12. Purification scheme for SMP-III
Figure 4.13 SDS polyacrylamide gel of SMP-III. Lane a- SMP-III, lane b- Bio-Rad protein standards, lane c- SMP-I
<table>
<thead>
<tr>
<th>Condition</th>
<th>Volume</th>
<th>Heavy Chain</th>
<th>Light Chain</th>
<th>Specific Activity</th>
<th>Total Activity</th>
<th>Folded</th>
<th>Total</th>
<th>Specific Activity</th>
<th>Total Activity</th>
<th>Folded</th>
</tr>
</thead>
</table>
| Assay Conditions: 50 mM Tris-HCl (pH 7.4), 1 mM DTT, 1 μM substrate, 30°C
| 2050         | 501    | 15          | 888         | 444              | 13             | 0.03    | 1       | 24                | 0.02           | 21      |
| 1050         | 210    | 101         | 354         | 177              | 85             | 0.2     | 21      | 50                | 30             | 20      |
| 62           | 118    | 88          | 48.8        | 93               | 0.1            | 9       | 19      | 27                | 0.3            | 24      |
| 10           | 321    | 15          | 77          | 247              | 0.9            | 36.2    | 33      | 270               | 0.9            | 33      |
| 16           | 1911   | 8           | 3.8         | 45.9             | 45.9           | 44.1    | 33      | 240               | 0.9            | 33      |
| 05           | 3324   | 2           | 8           | 0.8              | 0.8            | 0.8     | 0.8     | 0.8               | 0.8            | 0.8     |
| 02           | 1832   | 1           | 1           | 0.5              | 0.5            | 0.5     | 0.5     | 0.5               | 0.5            | 0.5     |

Table 4.1: Purification table for SMF-III
4.2 Purification of a myofibril associated phosphatase

SMP-III is prepared from the cytosolic fraction obtained from a low salt extraction of turkey gizzard smooth muscle. Recent studies have described the purification of myosin phosphatases from chicken and turkey gizzard myofibrils. These studies prompted an examination of the myofibril pellet following the extraction of the cytosolic phosphatases. About 75% of the HMM phosphatase activity remains bound to the myofibril after the initial extraction. For this reason the myofibril pellet was re-extracted with a high salt buffer and the myofibril phosphatase was purified. The purification scheme used was identical to that used for the purification of SMP-III except for the changes noted in section 3.1.3.

The myofibril extract was precipitated with 0-60% saturated ammonium sulfate. The pellet was dissolved in a minimum volume of buffer and was applied to a DEAE-Sephacel ion exchange column. The phosphatase activity was eluted from the column at 220-420 mM KCl which is similar to the concentration required to elute the cytosolic phosphatases from DEAE-Sephacel (figure 4.14). The fractions with phosphatase activity were pooled and concentrated by ammonium sulfate precipitation. The pellet was dissolved in a minimum volume of buffer and applied to a Sephacryl S-300 gel filtration column.

Chromatography on Sephacryl S-300 fractionated the phosphatase activity into two peaks, both with activity towards both MLC$_{20}$ and HMM (figure 4.15). The elution volumes for these peaks (860 and 1010 ml, respectively) are nearly identical to peaks A (850 ml) and B (1060 ml) from the chromatography of SMP-III on Sephacryl S-300 (figure 4.2). As was done during the purification of SMP-III, the S-300 eluate was assayed for
Figure 4.14. Elution profile for the chromatography of a myofibril bound phosphatase on DEAE-Sephadex. The column (5x8 cm) was run at 87 ml/hr, eluted with a linear 20-500 mM KCl gradient and fractions of 8.7 ml were collected. The fractions were assayed for phosphatase activity towards MLC$_{20}$ (□). The absorbance at 280 nm is shown (—).

MLC$_{20}$ activity in the presence of 10 mM Mg$^{2+}$ in order to identify the PP2C phosphatase activity. No Mg$^{2+}$ dependent activity was found suggesting that SMP-II (PP2C) does not bind to the myofibril. Western blot analysis of the phosphatase containing peaks showed cross-reactivity with anti-PP1 phosphatase monoclonal antibodies and not with anti-PP2A phosphatase or anti-PP2C phosphatase polyclonal antibodies suggesting that neither SMP-I nor SMP-II bind to the myofibril. These observations suggest that the two activity peaks obtained from Sephadryl S-300 are
analogous to SMP-III and SMP-IV obtained in the purification of the cytosolic phosphatases.

Figure 4.15. Elution profile for the chromatography of a myofibril bound phosphatase on Sephacryl S-300. The column (8x87 cm) was run at 80 ml/hr and 5.3 ml fractions were collected. The fractions were assayed for phosphatase activity towards MLC$_{20}$ (☐) and HMM (●). The absorbance at 280 nm is shown (−).

The first peak (analogous to SMP-III) was further purified by chromatography on Heparin-Sepharose (figure 4.16). One major peak with activity towards both MLC$_{20}$ and HMM was eluted from this column with 280-580 mM KCl.
Figure 4.16. Elution profile for the chromatography of a myofibril bound phosphatase on Heparin-Sepharose. The column (1.5x5 cm) was run at 100 ml/hr, eluted with a linear 20-800 mM KCl gradient and 3.8 ml fractions were collected. The fractions were assayed for phosphatase activity towards MLC$_{20}$ (□) and HMM (●). The absorbance at 280 nm is shown (-).

The phosphatase peak eluted from Heparin-Sepharose was chromatographed on thiophosphorylated MLC$_{20}$-Sepharose (figure 4.17). Owing to its much higher binding capacity, this resin was used instead of the thiophosphorylated HMM-Sepharose. Thiophosphorylated MLC$_{20}$-Sepharose has been used successfully in the purification of a myosin bound phosphatase (MBP) from chicken gizzards whereby MBP was eluted from thiophosphorylated HMM-Sepharose at ~200 mM KCl (Okubo et al.,
Thus a stepwise elution was used in the chromatography of the turkey gizzard myofibril phosphatase. Following the initial low salt wash to remove unbound proteins, the column was washed with 0.13 M KCl followed by a 1.0 M KCl elution. Two peaks of phosphatase activity were obtained, one in the 0.13 M wash and one in the 1 M KCl eluate. Three protein bands at 22, 38 and 130 kDa were observed in the SDS PAGE of the 1 M KCl eluate (figure 4.18). These three protein bands are also present in

![Graph showing elution profile](image)

**Figure 4.17.** Elution profile for thiophosphorylated HMM-Sepharose in the purification of a myofibril bound phosphatase. The column (1.5x14.5 cm) was washed with equilibration buffer followed by 0.13 M KCl and eluted with 1.0 M KCl. The column was run at 70 ml/hr and 4 ml fractions of flow through were collected while 1 ml fractions of the eluate were collected. The fractions were assayed for phosphatase activity HMM (●). The absorbance at 280 nm is shown (—).
the 0.13 M KCl wash, however, this fraction also contains many other protein bands (results not shown).

4.3 Subunit composition of SMP-III

The SDS gel shown in figure 4.13 shows three major protein bands are present in the purified preparation of SMP-III. Non-denaturing polyacrylamide gel electrophoresis was used to determine whether these protein bands were subunits of SMP-III.

4.3.0 Non-denaturing polyacrylamide gel electrophoresis

Purified SMP-III was electrophoresed on a non-denaturing polyacrylamide tube gel. The gel was sliced into 2 mm segments and the segments were eluted overnight. The eluates were assayed for phosphatase activity and the active fractions were subjected to SDS PAGE. Figure 4.19a shows the phosphatase activity of the eluates. One peak of phosphatase activity was observed with the highest activity found in fraction 9. Lanes 1-6 of the silver stained SDS PAGE shown in figure 4.19b correspond to slices 6-11. Only the 38 kDa band is found in all fractions containing phosphatase activity. Other protein bands were observed on the silver stained gel; however, these do not correlate with phosphatase activity. This experiment was repeated five times and the same result was obtained each time. We, therefore, conclude that the 38 kDa protein is SMP-III. However, this does not preclude the possibility that SMP-III has regulatory subunits which dissociate during the experimental manipulation.

Since only the 38 kDa protein was found to correlate with phosphatase activity, the relationship of the two other proteins (130 and 22
Figure 4.18 SDS polyacrylamide gel of SMP-III$_M$. Lane a- SMP-III$_M$, lane b-SMP-I, lane c- Bio-Rad protein standards.
Figure 4.19 Non-denaturing polyacrylamide gel electrophoresis of SMP-III. Eluates from slices of a 3.5% polyacrylamide tube gel were assayed for phosphatase activity (A) towards MLC\(_{20}\) (●) and HMM (○). Eluates with phosphatase activity were subjected to SDS PAGE (B). Lanes 1-6: gel slices 6-11; lane 7: SMP-I; lane 8: Bio-Rad protein standards.
kDa) with the phosphatase (38 kDa) was investigated. The molecular weight of SMP-III determined by gel filtration chromatography was 390 kDa is consistent with a multisubunit protein, a highly asymmetric protein, or both. SMP-III may be composed of 10 subunits of the 38 kDa catalytic subunit. Alternatively, the high molecular weight could be explained if the 22 and 130 kDa proteins were subunits of SMP-III, however, the dissociation of these proteins when subjected to non-denaturing electrophoresis suggests they have very low affinity for one another.

In order to clarify this point, partially-purified preparations of SMP-III were analysed for their subunit composition by immunoprecipitation.

4.3.1 Immunoprecipitation

Several attempts were made to immunoprecipitate the SMP-III catalytic subunit and any accompanying regulatory subunits with the anti-PP1 monoclonal antibody. Partially-purified SMP-III was incubated with anti-PP1 monoclonal and the resulting antibody-phosphatase complex was precipitated with pansorbin and centrifuged. Since the amount of precipitated protein was too small to be visualized by Coomassie Blue or silver-stained gels, other methods of detection were employed. The 38 kDa subunit was visualized by Western blot with anti-PP1 monoclonal antibodies (figure 4.20a). As will be discussed later, the 130 and 22 kDa proteins that co-purify with SMP-III can be phosphorylated by PK-C (section 4.11). We, therefore, phosphorylated the dissolved pellet using [γ-32P] ATP and the labeled co-precipitates were subjected to SDS PAGE and visualized by autoradiography (figure 4.20b). A Western blot of the immunoprecipitate shows a small amount of SMP-III catalytic subunit was
precipitated (figure 4.20a). Figure 4.20b shows an autoradiograph of the immunoprecipitate. Lane 1 shows that three high molecular weight bands (200, 130 and 68 kDa) are enriched in the immunoprecipitate but the 22 kDa protein was not observed. The co-precipitation of the 130 kDa protein supports the hypothesis that this protein is a subunit of SMP-III. The other two proteins (200 and 68 kDa) may also also be subunits, however, since these proteins are not observed in the purified SMP-III preparations there relationship with the 38 kDa phosphatase remains uncertain.

If indeed SMP-III has regulatory subunits which are loosely bound to the catalytic subunit, covalent cross-linking of these subunits before immunoprecipitation may aid their identification. Thus, cross-linking closely associated proteins with a reversible chemical crosslinker was attempted. Following immunoprecipitation, the crosslinks were to be cleaved with mercaptoethanol and boiling according to the manufacturers instructions. However, the crosslinks proved to be difficult to cleave. Partial cleavage of the crosslinks was achieved at concentrations of mercaptoethanol exceeding 1 M. Furthermore, no immunoprecipitation was observed when chemical cross-linking was performed. Steric interference resulting from the crosslinking may have prevented the binding of the antibody to its epitope.

4.4 Enzymatic properties of SMP-III

To characterize and compare the properties of SMP-III to other protein phosphatases, the substrate specificity and kinetic properties of SMP-III were examined
Figure 4.20 Immunoprecipitation of SMP-III. SMP-III was immunoprecipitated with anti-PP1C monoclonal antibody (MAb). The precipitate was subjected to western blot analysis (A- lanes a, b, and c are replicates) or autoradiography after phosphorylation with PK-C (B- lane a- SMP-III, MAb and pansorbin, lane b- SMP-III and pansorbin, lane c- MAb and pansorbin, lane d- SMP-III and MAb, lane e- SMP-III, lane f- total SMP-III, lane g- MAb, lane h- pansorbin, lane i- PK-C alone).
4.4.0 Substrate specificity

The substrate specificity of SMP-III was determined toward contractile and non-contractile proteins (table 4.2). SMP-III is highly specific for smooth muscle MLC$_{20}$ either free or when complexed in HMM or intact myosin. SMP-III has little or no activity towards the other substrates tested.

Table 4.2. Substrate specificity of SMP-III

<table>
<thead>
<tr>
<th>Substrate (1 μM)</th>
<th>relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLC$_{20}$</td>
<td>100</td>
</tr>
<tr>
<td>HMM</td>
<td>108</td>
</tr>
<tr>
<td>Myosin</td>
<td>174</td>
</tr>
<tr>
<td>Histone IIA</td>
<td>no activity</td>
</tr>
<tr>
<td>Phosphorylase $a$</td>
<td>2.0</td>
</tr>
<tr>
<td>Phosphorylase kinase</td>
<td>no activity</td>
</tr>
</tbody>
</table>

The rate of dephosphorylation of MLC$_{20}$ was defined as 100 % activity. Values shown are the average of values from 4 experiments. Assay conditions: 50 mM Tris-HCl (pH 7.4), 1 mM DTT, 1 μM substrate, 30 °C.

4.4.1 Kinetic properties

The Lineweaver-Burk plots were used to determine the $K_M$ and $V_{max}$ for SMP-III using MLC$_{20}$ and HMM as substrates (figure 4.21). The
$K_M$ was 2 and 5 $\mu$M for MLC$_{20}$ and HMM, respectively. The $V_{max}$ was 210 and 670 nmol/min/mg for MLC$_{20}$ and HMM, respectively.

4.4.2 pH profile for SMP-III

The activity of SMP-III towards MLC$_{20}$ and HMM at different pHs was determined. The buffers used for the different pH were sodium acetate for pH 5 and 5.5, sodium phosphate for pH 6 and 6.5, MOPS-NaOH for pH 6.5-7.5, Tris-HCl for pH 7.0-8.5, and glycine-HCl for pH 8.5 and 9. Figure 4.22 shows that SMP-III has a broad pH specificity ranging between pH 7 and 8.5.

4.5 Classification of SMP-III

Ingebritsen and Cohen (1983) have proposed a scheme for the classification of serine/threonine phosphatases based on two criteria. The first criterion is the sensitivity of the phosphatase to inhibition by heat stable inhibitor-2. The effect of heat stable inhibitor-2 on SMP-III activity towards MLC$_{20}$ and HMM was determined and shown in figure 4.23. The 34 kDa form of rabbit skeletal muscle PP1C (referred to here as PP1C') and SMP-I were included in this study as controls for type 1 and type 2A protein phosphatases, respectively. Neither PP2A (results not shown) nor SMP-III are affected by inhibitor-2 (figure 4.23) suggesting that SMP-III is a type 2 protein phosphatase.
Figure 4.21. Double reciprocal plots of velocity of the reaction against the concentration of substrate using A. MLC_{20} (□) and B. HMM (●) as substrates.
Figure 4.22. The effect of pH on the activity of SMP-III towards MLC\textsubscript{20} (□), HMM (●).

The second criterion is the activity of the phosphatase towards phosphorylase kinase. Phosphorylase kinase is composed of four subunits. Two subunits (α and β) are phosphorylated by cAMP-dependent protein kinase. Type 1 phosphatases preferentially dephosphorylate the α subunit while type 2 phosphatases dephosphorylate the β subunit. \[^{32}\text{P}\] labeled phosphorylase kinase was incubated with SMP-III, aliquots were taken at various times and subjected to SDS PAGE and autoradiography (figure 4.24). No dephosphorylation of either the α or β subunits was observed. In order to ensure that SMP-III was active under the assay conditions, the same experiment was carried out but MLC\textsubscript{20} was included in the reaction mix as a control. It was observed that while MLC\textsubscript{20} was dephosphorylated, the α and β subunits of phosphorylase kinase were not dephosphorylated (results not shown).
Figure 4.23. Effect of heat stable inhibitor-2 on the activities of SMP-III (\(\phi\)) and rabbit muscle PP-1C' (\(\Box\)) towards MLC\(_{20}\) (A) and HMM (B).
Figure 4.24 Dephosphorylation of phosphorylase kinase. Phosphorylase kinase was incubated with PP-1C', PP2A (SMP-I) (A) or SMP-III (B) and aliquots were removed at various times from 0 to 60 minutes (lane headings) The reaction was stopped by adding SDS sample buffer and boiling. The samples were electrophoresed on 12.5 % polyacrylamide gels and the gels were autoradiographed.
A summary of the classification of SMP-III shows that this phosphatase does not fit the scheme proposed by Ingebritsen and Cohen (table 4.3).

Table 4.3. Classification of SMP-III

<table>
<thead>
<tr>
<th>Effect of inhibitor-2</th>
<th>Dephosphorylation of phosphorylase kinase</th>
<th>Requirement for divalent cations</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP1</td>
<td>inhibited</td>
<td>β subunit</td>
</tr>
<tr>
<td>PP2A</td>
<td>not inhibited</td>
<td>α subunit</td>
</tr>
<tr>
<td>PP2B</td>
<td>not inhibited</td>
<td>α subunit</td>
</tr>
<tr>
<td>PP2C</td>
<td>not inhibited</td>
<td>α subunit</td>
</tr>
<tr>
<td>SMP-III</td>
<td>not inhibited</td>
<td>neither</td>
</tr>
</tbody>
</table>

Type 1 and type 2 phosphatases can also be differentiated by a number of other properties not used in the classification of the enzymes. One of these properties is the sensitivity of the phosphatases to okadaic acid. Type 2A enzymes are about 100 times more sensitive to okadaic acid than type 1 phosphatases. Type 2B phosphatases are inhibited by 1000-fold higher concentrations than type 2A phosphatases while type 2C phosphatases are resistant to inhibition by okadaic acid. SMP-III was tested for its sensitivity to okadaic acid. Figure 4.25 shows a comparison of the dose dependent inhibition of SMP-III, SMP-I (PP2A) and PP1C'. IC$_{50}$
values of 10 nM and 1 μM for PP2A and PP1, respectively, were observed. The inhibition curves show that SMP-III is inhibited by concentrations similar to those that inhibit type 1 phosphatase.

4.5.0 Structural properties of serine/threonine phosphatases

The different serine/threonine phosphatases can be distinguished by their primary structure. This is reflected by differences in their immunoreactivity and their amino acid sequence. SMP-III was examined by western blot analysis to determine its structural similarity with type 1, type 2A and type 2C protein phosphatases. Type 1 monoclonal antibodies were obtained from Dr. J. Vandenheede, Katholieke Universiteit, Leuven, Belgium. Western blot with anti-PP1C polyclonal antibodies was performed by Dr. D. Brautigan, Brown University while western blots with anti-PP1C C-terminal polyclonal antibodies were performed by Dr. D. Brautigan and Dr. K. Schlender, University of Ohio. The anti-PP1C polyclonal antibodies were raised against PP1C' (Brautigan et al., 1985) while the anti C-terminal polyclonal antibodies were raised against residues 312-326 of PP1α (Dr. D. Brautigan, personal communication) and residues 319-330 of PP1α (Wang et al., 1992). These peptides are about 50% identical with the corresponding sequences of the other three isoforms of PP1C. Anti-PP2A antibodies were raised against SMP-I holoenzyme and the catalytic subunit of SMP-I while anti-PP2C antibodies were raised against SMP-II. SMP-III cross-reacts with the various type 1 antibodies tested but not with either the type 2A or type 2C antibodies indicating that SMP-III has structural similarity to type 1 phosphatases. The results of this analysis are summarized in table 4.4.
Figure 4.25. The effect of okadaic acid on activity of SMP-III (◆), PP2A (○) and PP1C' (□) towards MLC$_{20}$ (A) and HMM (B).
Figure 4.26 shows a western blot of SMP-III, SMP-IV, PP1α, PP1γ1, PP1γ2, PP1δ and rabbit muscle PP1C. This result shows that the catalytic subunits SMP-III and SMP-IV have the same molecular weight (38 kDa) as the recombinant phosphatases.

Table 4.4. Summary of antibody cross-reactivity of SMP-III

<table>
<thead>
<tr>
<th>Antibody</th>
<th>cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-PP2A1 polyclonal</td>
<td>no</td>
</tr>
<tr>
<td>anti-PP2A catalytic subunit polyclonal</td>
<td>no</td>
</tr>
<tr>
<td>anti-PP2C polyclonal</td>
<td>no</td>
</tr>
<tr>
<td>anti-PP1C monoclonal</td>
<td>yes</td>
</tr>
<tr>
<td>anti-PP1C polyclonal</td>
<td>yes</td>
</tr>
<tr>
<td>anti-PP1C C-terminal peptide polyclonal</td>
<td>yes</td>
</tr>
</tbody>
</table>

The primary sequence for type 1 and type 2 phosphatases has been determined through cDNA and amino acid sequencing. Several isoforms of each type have been identified. Amino acid sequences of proteolytic peptides of SMP-III were used to further characterize the phosphatase. In order to obtain an amino acid sequence several obstacles had to be overcome. The first of these was to obtain a sufficient amount of material for the sequencing procedure. Since only 30 µg of SMP-III is obtained from 360 g of smooth muscle, the amount of gizzard smooth muscle used in the preparation was increased to 1000 g. The increase in starting material
Figure 4.26 Western blot analysis of smooth muscle myosin phosphatases and recombinant type 1 phosphatases. Lane a- SMP-III, lane b- SMP-IV, lane c- PP1δ, lane d- PP1α, lane e- PP1γ1, lane f- PP1γ2, lane g- PP1C. 
necessitated some modifications to the columns used in the purification procedure. The volume of DEAE-Sephacel and Heparin-Sepharose had to be increased to accommodate the increased amount of protein. Two applications to the Sephacryl S-300 column was also necessary.

In order to obtain an amino acid sequence, SMP-III must be free of contaminants. This posed a problem since many additional minor bands are often seen in the SMP-III preparations. Two of the commonly observed bands migrate on SDS PAGE like proteins of 38 and 44 kDa. This pattern is similar to that of tropomyosin. Western blot analysis with antitropomyosin polyclonal antibodies confirmed the identity of the bands as tropomyosin. The lower of the two bands co-migrates with the catalytic subunit of SMP-III on SDS PAGE (figure 4.27). It has been previously reported that the migration of tropomyosin on SDS PAGE is altered by the presence of urea. We used this property to separate SMP-III from tropomyosin. Figure 4.27 shows the separation of SMP-III from tropomyosin by using SDS PAGE containing 2 M urea. In order to obtain sufficient SMP-III for amino acid sequencing, preparative electrophoresis of SMP-III was carried out on 2 M urea SDS PAGE and transferred to immobilon. The proteins were visualized by staining with Ponceau S and the SMP-IIIc band was cut out and sent to Harvard Microchem where the phosphatase was exhaustively digested with trypsin and the resulting peptides were separated by HPLC. Two proteolytic peptides were obtained and sequenced (figure 4.28). The amino acid sequence of one peptide is found to correspond to residues 111-121 of PP1δ. This sequence is found in all type 1 isoforms reported to date. The other peptide sequence obtained corresponds to residues 221-233, a sequence found only in the type 1δ
Figure 4.27 Western blot analysis of SMP-III after SDS (lanes a-d) and SDS-urea (lanes e-h) PAGE. PP1C' was applied to lanes a and f; SMP-III was applied to lanes b, c, e, and g; tropomyosin was applied to lanes d and h. Lanes a, b, e, and f were blotted with anti-PP1C monoclonal antibodies, lanes c, d, g, and h were blotted with anti-tropomyosin polyclonal antibodies.
isoform. This sequence differs from corresponding sequences in the other isozymes by two amino acids.

![Amino acid sequence of two proteolytic peptides from SMP-III](image)

Figure 4.28. Amino acid sequence of two proteolytic peptides from SMP-III

### 4.6 Classification of the myofibril bound phosphatase

Classification of the myofibril bound phosphatase was also attempted. As with SMP-III, the myofibril bound phosphatases cannot be classified according to the scheme of Ingebritsen and Cohen (1983). The myofibril phosphatase was not affected by heat stable inhibitor 2 and did not dephosphorylate either the α or β subunits of phosphorylase kinase. As mentioned earlier, this phosphatase cross-reacted with the anti-type 1 monoclonal antibody. These and other properties such as the characteristic chromatographic profiles and myosin binding are very much like those described for SMP-III suggesting that these enzymes might be
identical. Thus, the myofibril bound phosphatase will be referred to as SMP-III\textsubscript{M} to denote its similarity to SMP-III and its myofibril origin.

4.7 Tryptic digestion studies

It has been reported that the catalytic subunit of PP1 is susceptible to proteolysis and that this degradation alters the activity of the enzyme. We decided to determine if limited tryptic digestion would affect SMP-III activity towards MLC\textsubscript{20} and HMM.

A time course of tryptic digestion is shown in figure 4.29. SMP-III was incubated with trypsin and aliquots were removed at various time points and added to soybean trypsin inhibitor to stop the reaction. Proteolysis was observed to stimulate SMP-III activity towards both MLC\textsubscript{20} and HMM to a maximum of 175 and 200 % of control, respectively. Maximum stimulation was observed in the first 3 minutes of digestion. The level of stimulation decreases with prolonged digestion.

In order to determine if the change in the activity of SMP-III was due to the proteolysis of the catalytic subunit, we examined the phosphatase by western blot analysis at various time points during the digestion. The effect of digestion on the catalytic subunit of SMP-IV was also examined. After 5 minutes of digestion the 38 kDa catalytic subunit of both SMP-III and IV were converted to a 37 kDa proteolytic product (figure 4.30). Continued digestion produces a 34 kDa species which migrates at about the same rate as PP1C'. Western blot analysis has also been performed on phosphatases after longer periods of digestion. These blots indicate that the 34 kDa form becomes the dominant species by 45 minutes of digestion.
Figure 4.29. Effect of digestion on the activity of SMP-III towards MLC$_{20}$ (A) and HMM (B). digestion (●), control (□).
The time course of SMP-III digestion was also monitored using anti-PP-1C C-terminal antibodies (figure 4.30b). On this blot the 37 kDa species appears by 1 minute and is the dominant species after 5 minutes of digestion. The amount of the 37 kDa species decreases as digestion progressed but the 34 kDa species is not visible on the blot. This indicates that the peptide cleaved in the 37 to 34 kDa conversion is from the C-terminal portion of the molecule.

In order to further characterize the effect of digestion on phosphatase activity the $K_M$ and $V_{max}$ of SMP-III before and after digestion were determined. The western blot of the time course of digestion shows that most of the phosphatase has been converted to a 37 kDa species by 5 minutes of digestion (figure 4.30). We determined the effect of this conversion on the kinetic properties of SMP-III (table 4.5). The $V_{max}$ of SMP-III for both MLC$_{20}$ and HMM is increased while the $K_M$ is unchanged by the digestion.

In view of these effects we set out to determine whether limited tryptic digestion would also affect other properties of SMP-III. The first property examined was the effect of heat stable inhibitor 2 on SMP-III activity. For comparison, SMP-IV and SMP-IIIc were included in this study. Figure 4.31 shows the effect of different times of digestion (0, 1, and 45 minutes) on the sensitivity of SMP-III to inhibitor 2. As the time of digestion is increased from 1 to 45 minutes SMP-III becomes more sensitive to inhibition by inhibitor 2. The enzyme's sensitivity to inhibitor 2 begins to approach that of rabbit skeletal muscle PP1C' which was used as a control. Recombinant PP18 was also tested for sensitivity to inhibitor 2 and it displayed a similar degree of inhibition as observed for the rabbit skeletal muscle PP1C' (results not shown).
A. Western blot with anti-PP1C monoclonal antibodies

B. Western blot with anti-PP1C carboxyl terminus peptide polyclonal antibodies

Figure 4.30 Western blot analysis of the time course of tryptic digestion. SMP-III and IV were incubated with trypsin and aliquots were removed at various times (lane headings) from 0 to 60 minutes. The reaction was stopped by addition to soybean trypsin inhibitor solution. The samples were analysed by western blot using anti-PP1C monoclonal antibodies (A) and anti-PP1C C-terminal polyclonal antibodies. PP1C' was included as a control.
Table 4.5. Summary of the effect of digestion on the $K_m$ and $V_{max}$ of SMP-III

<table>
<thead>
<tr>
<th></th>
<th>MLC$_{20}$</th>
<th>HMM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$, $\mu$M</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>undigested</td>
<td>6.7</td>
<td>182</td>
</tr>
<tr>
<td>5 min digest</td>
<td>6.7</td>
<td>286</td>
</tr>
</tbody>
</table>

Assay conditions: 50 mM Tris-HCl (pH 7.4), 1 mM DTT, substrate, 30 $^\circ$C

Figure 4.30 shows that limited proteolysis results in the conversion of the 38 kDa catalytic subunit of SMP-III into a 34 kDa product with similar migration on SDS PAGE as PP1C'. However, this proteolytic product is less sensitive to inhibitor 2 than is PP1C' suggesting that regulatory subunits may be involved. In order to further study this problem, we isolated the catalytic subunit of SMP-III (SMP-IIIc) by ethanol precipitation. SMP-III was precipitated in 4 volumes of ice cold 95% ethanol. The solution was centrifuged and the pellet was extracted with buffer C (20 mM KCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF). The solution was centrifuged, supernatant was removed and dialyzed against 50% glycerol. A SDS PAGE of SMP-IIIc is shown in figure 4.32. Like SMP-III, SMP-IIIc is insensitive to inhibitor 2. Digestion of SMP-IIIc for 30 minutes resulted in
Figure 4.31. Effect of limited tryptic digestion of SMP-III on the sensitivity of SMP-III to inhibitor-2. The activity towards MLC$_{20}$ (A) and HMM (B) was determined after no digestion (○), 1 minute digestion (△) and 45 minute digestion (●). FP1C' (□) is shown as control.
Figure 4.32 SDS polyacrylamide gel of SMP-IIIc. Lane a- SMP-IIIc, lane b- SMP-I, lane c- Bio-Rad protein standards.
a phosphatase with similar sensitivity to inhibitor 2 as seen with PP1C' (figure 4.33). When products of shorter digestion times (10 and 20 minutes) were examined, intermediate levels of inhibitor 2 sensitivity were observed (results not shown).

SMP-IV is another turkey gizzard smooth muscle myosin phosphatase. This enzyme was previously shown to be insensitive to inhibitor 2 (Pato and Kerc, 1985). We subjected SMP-IV to limited tryptic digestion and examined the product for sensitivity to inhibitor 2. Figure 4.34 shows that limited proteolysis also converts SMP-IV into an inhibitor 2 sensitive phosphatase.

The effect of digestion on the activity of SMP-III towards phosphorylase kinase was also examined (figure 4.35b). SMP-III was incubated with trypsin and aliquots were removed after 0 (no trypsin added), 5 and 40 minutes of digestion and added to soybean trypsin inhibitor to stop the reaction. As shown previously in section 4.4, undigested SMP-III does not dephosphorylate phosphorylase kinase. Following 5 minutes of proteolysis, SMP-III becomes active towards the β subunit of phosphorylase kinase. Proteolysis of SMP-III for 40 minutes increases the rate at which it dephosphorylates the β subunit.

When originally isolated, SMP-IV showed activity towards the β subunit of phosphorylase kinase (Pato and Kerc, 1985). However, more recent preparations did not dephosphorylate phosphorylase kinase. We subjected SMP-IV to limited tryptic digestion and examined the phosphorylase kinase activity of the products after 0, 5 and 40 minutes of digestion (figure 4.35c). As seen with SMP-III, limited proteolysis of SMP-IV results in the activation of the phosphatase towards the β subunit of
A.

![Graph A]

B.

![Graph B]

Figure 4.33. Effect of limited tryptic digestion of SMP-IIIc on the sensitivity of SMP-IIIc to inhibitor 2. The activity towards MLC$_{20}$ (A) and HMM (B) was determined after no digestion (○) and 30 minute digestion (●). PP1C' (□) is shown as control.
Figure 4.34. Effect of limited tryptic digestion of SMP-IV on the sensitivity of SMP-IV to inhibitor 2. The activity towards MLC_{20} (A) and HMM (B) was determined after no digestion (○) and 45 minute digestion (●). PP1C' (□) is shown as control.
A. Control
   a. Type 1  b. Type 2A
   0 5 10 30 60   0 5 10 30 60 min.

B. SMP-III
   a. undigested  b. 5 min. digest  c. 40 min. digest
   0 5 10 30 60   5 10 30 60   5 10 30 60 min.

C. SMP-IV
   a. undigested  b. 5 min. digest  c. 40 min. digest
   0 5 10 30 60   5 10 30 60   5 10 30 60 min.

Figure 4.35 Effect of tryptic digestion on the dephosphorylation of phosphorylase kinase. Phosphorylase kinase was dephosphorylated by PP1C' (A.a) and PP2A (A.b); SMP-III (B.a), 5 minute (B.b) and 40 minute (B.c) digests of SMP-III; SMP-IV (C.a), and 5 minute (C.b) and 40 minute (C.c) digests of SMP-IV. Aliquots were removed at various times from 0 to 60 minutes (lane headings) and subjected to SDS PAGE. The gels were autoradiographed.
phosphorylase kinase. This suggests that the SMP-IV used during the original characterization may have undergone proteolysis during purification.

4.8 Regulation of SMP-III

The effect of divalent cations, nucleotides, NaF, and KCl on phosphatase activity has been determined for many of the serine/threonine phosphatases described to date. In order to compare SMP-III with other phosphatases reported in the literature, the effect of these reagents on SMP-III activity was determined.

4.8.0 Effect of divalent cations on SMP-III

The activity of a number of phosphatases is affected by divalent cations. These include type 2B and type 2C enzymes which have an absolute requirement for Ca\(^{2+}\) and Mg\(^{2+}\), respectively. In addition, the activity of several type 1 phosphatases are affected by divalent cations. The effect of divalent cations on the activity of SMP-III towards MLC\(_{20}\) and HMM was determined.

SMP-III activity towards both MLC\(_{20}\) and HMM was stimulated by Mn\(^{2+}\) (figure 4.36). Activity towards MLC\(_{20}\) was stimulated to a maximum of 400\% of control at 5 mM Mn\(^{2+}\) while activity towards HMM was stimulated to a maximum of 200\% of control at 2.5 mM Mn\(^{2+}\). SMP-III activity towards both MLC\(_{20}\) and HMM decreased with increasing concentrations of Mn\(^{2+}\).

SMP-III activity is also affected by Mg\(^{2+}\) (figure 4.37). Activity towards HMM is stimulated to a maximum of 130\% of control at 5 mM Mg\(^{2+}\). At concentrations higher than 11 mM, SMP-III activity is inhibited
in a concentration-dependent manner. Mg$^{2+}$ inhibits SMP-III activity towards MLC$_{20}$ in a concentration dependent manner.

The effect of Co$^{2+}$ on SMP-III activity was also determined (figure 4.38). Phosphatase activity was inhibited with IC$_{50}$ values of 100 and 250 μM for MLC$_{20}$ and HMM, respectively.

The effect of Ca$^{2+}$, Cu$^{2+}$, and Ni$^{2+}$ on the activity of SMP-III was also determined. Cu$^{2+}$ and Ni$^{2+}$ are potent inhibitors of SMP-III activity with similar IC$_{50}$ values to those of Co$^{2+}$. The IC$_{50}$ values for Cu$^{2+}$ are 200 and 600 μM for MLC$_{20}$ and HMM, respectively, while those for Ni$^{2+}$ are 200 and 400 μM for MLC$_{20}$ and HMM, respectively. Ca$^{2+}$ had no effect on SMP-III activity at concentrations up to 1mM in the absence or presence of 0.5 μM calmodulin.
Figure 4.37. Effect of Mg\(^{2+}\) on the activity of SMP-III towards MLC\(_{20}\) (□) and HMM (●).

Figure 4.38. Effect of Co\(^{2+}\) on the activity of SMP-III towards MLC\(_{20}\) (□) and HMM (●).
To further characterize the effect of divalent cations on myosin phosphatase activity, SMP-IIIc was also studied.

As seen with SMP-III, the activity of SMP-IIIc is also stimulated by Mn$^{2+}$ (figure 4.39). At 1 mM Mn$^{2+}$ SMP-IIIc activity is stimulated to 150 and 200 % towards MLC$_{20}$ and HMM, respectively. High concentrations of Mn$^{2+}$ inhibits the activity of SMP-IIIc towards both substrates.

![Graph showing the effect of Mn$^{2+}$ on the activity of SMP-IIIc](image)

**Figure 4.39.** Effect of Mn$^{2+}$ on the activity of SMP-IIIc towards MLC$_{20}$ (□) and HMM (●).

The effect of Mg$^{2+}$ on SMP-IIIc activity was also determined. Low concentrations of Mg$^{2+}$ causes a small degree of stimulation of SMP-IIIc towards MLC$_{20}$ while the same Mg$^{2+}$ concentrations inhibit SMP-III (figure 4.40). SMP-IIIc activity towards HMM is stimulated to 170 % at 10 mM Mg$^{2+}$. 
Figure 4.40. Effect of Mg$^{2+}$ on the activity of SMP-IIIc towards MLC$_{20}$ (□) and HMM (●).

Co$^{2+}$ inhibited SMP-IIIc activity towards both MLC$_{20}$ and HMM with IC$_{50}$ values of 30 and 200 µM, respectively (figure 4.41). This is a similar to the inhibition seen with SMP-III.

As seen with SMP-III, Ca$^{2+}$ concentrations up to 1.0 mM had no effect on the activity of SMP-IIIc (results not shown).

4.8.1 Effect of nucleotides on SMP-III activity

The effect of ATP, GTP, GDP, GMP and cGMP on SMP-III activity was determined. Table 4.6 shows that SMP-III activity towards MLC$_{20}$ and HMM is inhibited to varying degrees by ATP, GTP and GDP. GTP is the most potent inhibitor followed by GDP then ATP. GMP and cGMP have no effect on SMP-III activity over the concentration range tested.
Figure 4.41 Effect of Co$^{2+}$ on the activity of SMP-IIIc towards MLC$_{20}$ (□) and HMM (●).

4.8.2 Other effectors of SMP-III activity

The effect of a common phosphatase inhibitor, NaF, on SMP-III activity was also determined. NaF inhibited SMP-III activity with an IC$_{50}$ value of 20 mM for both MLC$_{20}$ and HMM.

The effect of KCl on SMP-III activity was determined. SMP-III activity was stimulated to a maximum of 130 % of control at 50 mM KCl when MLC$_{20}$ was used as substrate. KCl became inhibitory at higher concentrations with an IC$_{50}$ of 220 mM. A similar profile was obtained when HMM was used as substrate. SMP-III activity was stimulated to a maximum of 220 % of control at 100 mM KCl while the IC$_{50}$ for KCl was 300 mM.
Table 4.6. Summary of the effects of nucleotides on SMP-III activity

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>IC$_{50}$, mM</th>
<th>MLC$_{20}$</th>
<th>HMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>6</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>0.4</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>GDP</td>
<td>1.5</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>GMP</td>
<td>no effect</td>
<td>no effect</td>
<td></td>
</tr>
<tr>
<td>cGMP</td>
<td>no effect</td>
<td>no effect</td>
<td></td>
</tr>
</tbody>
</table>

Assay conditions: 50 mM Tris-HCl (pH 7.4), 1 mM DTT, substrate, 30 °C

4.9 Myosin binding properties of myosin phosphatases

The main objective of this project was to study the phosphatase involved in the dephosphorylation of myosin. To this end, we have purified two myosin phosphatases, SMP-III and SMP-III$_M$. Since myosin is the primary substrate for the phosphatases we set out to study the binding of these phosphatases to myosin and the factors that might affect the binding. SMP-IIIc, SMP-IV and PP1δ were also included in this study. The phosphatases were incubated with various concentrations of myosin and the mixture was centrifuged at ~100,000 rpm in a Beckman airfuge to pellet the myosin. The phosphatase activity of the supernatant represents the unbound fraction. The reciprocal of the fraction bound was plotted against
the reciprocal of the myosin concentration. The point at which the curve intersects the x-axis is defined as the $K_{\text{binding}}$ (listed in table 4.7). SMP-III and SMP-III_M showed identical $K_{\text{binding}}$ at $1.9 \times 10^6$ M$^{-1}$. The $K_{\text{binding}}$ for SMP-IV was slightly lower at $1.1 \times 10^6$ M$^{-1}$ while that of SMP-IIIc was 10 fold lower at $1.9 \times 10^5$ M$^{-1}$. PP1δ showed the highest $K_{\text{binding}}$ at $5.1 \times 10^6$ M$^{-1}$.

Table 4.7. $K_{\text{binding}}$ for several myosin phosphatases and PP1δ to myosin

<table>
<thead>
<tr>
<th>phosphatase</th>
<th>$K_{\text{binding}}$, M$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP-III</td>
<td>$1.9 \times 10^6$</td>
</tr>
<tr>
<td>SMP-IIIc</td>
<td>$1.9 \times 10^5$</td>
</tr>
<tr>
<td>SMP-IV</td>
<td>$1.1 \times 10^6$</td>
</tr>
<tr>
<td>SMP-III_M</td>
<td>$1.9 \times 10^6$</td>
</tr>
<tr>
<td>PP1δ</td>
<td>$5.1 \times 10^6$</td>
</tr>
</tbody>
</table>

We examined the effect of a number of reagents on the binding properties of SMP-III. The following is a list of reagents that had no measurable effect on the $K_{\text{binding}}$: Ca$^{2+}$, Mg$^{2+}$, EGTA, EDTA, ATP.

Of all the reagents tested, only GTP changed the $K_{\text{binding}}$ of SMP-III for myosin. Thus the effect of several different concentrations of GTP were examined. A concentration dependent increase in $K_{\text{binding}}$ was observed with a plateau reached at 0.5 mM. Increasing the GTP
concentration to 2 mM produced no further change in the $K_{\text{binding}}$. Figure 4.42 shows that 0.5 mM GTP increases the $K_{\text{binding}}$ from $1.9 \times 10^6$ M$^{-1}$ to $2.6 \times 10^6$ M$^{-1}$. The effect of 0.5 mM GTP on $K_{\text{binding}}$ of SMP-IIIc was also determined. Similar to the result obtained for SMP-III, the affinity of SMP-IIIc for myosin was increased from $1.9 \times 10^5$ to $7.7 \times 10^5$ M$^{-1}$ in the presence of 0.5 mM GTP.

![Graph showing the effect of GTP on myosin binding properties of SMP-III](image)

Figure 4.42. The effect of GTP on the myosin binding properties of SMP-III: no GTP (□), 0.5 mM GTP (●).

### 4.10 Role of GTP in the regulation of myosin phosphatase activity

Several recent studies have implicated GTP in the regulation of smooth muscle relaxation. For this reason, the effect of GTP on different myosin phosphatases was examined.

Table 4.8 lists the $IC_{50}$ values of GTP for SMP-III, SMP-IIIc, SMP-III$_M$, SMP-IV, PP1δ, PP1α, PP1γ1, and PP1γ2. The activities of the smooth muscle
myosin phosphatases are inhibited to about the same extent by GTP. However, the recombinant phosphatases are much more sensitive to GTP inhibition with IC$_{50}$ values about 100 times lower than those observed for the purified smooth muscle phosphatases. The effect of GTP$_{y}$S on SMP-III activity was also examined, however, no difference from the effect of GTP was observed.

Table 4.8. The effect of GTP on the activities of several myosin phosphatases towards MLC$_{20}$ and HMM

<table>
<thead>
<tr>
<th>phosphatases</th>
<th>IC$_{50}$, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MLC$_{20}$</td>
</tr>
<tr>
<td>SMP-III</td>
<td>400</td>
</tr>
<tr>
<td>SMP-IIIc</td>
<td>100</td>
</tr>
<tr>
<td>SMP-IV</td>
<td>600</td>
</tr>
<tr>
<td>SMP-III$_{M}$</td>
<td>300</td>
</tr>
<tr>
<td>PP1α</td>
<td>5</td>
</tr>
<tr>
<td>PP1γ$_{1}$</td>
<td>5</td>
</tr>
<tr>
<td>PP1γ$_{2}$</td>
<td>5</td>
</tr>
<tr>
<td>PP1δ</td>
<td>5</td>
</tr>
</tbody>
</table>

Assay condition: 50 mM Tris-HCl (pH 7.4), 1 mM DTT, substrate, 30 °C
The effect of pH on the inhibitory effect of GTP was determined. This is illustrated graphically in figure 4.43 which shows that at pH 7.4 GTP has an IC₅₀ of 0.4 mM for both MLC₂₀ and HMM whereas incubation at pH 7.0 decreases the IC₅₀ to 0.15 mM for both MLC₂₀ and HMM.

The inhibitory effect of GTP on binding and activity led us to question the mechanism through which GTP was acting. We first postulated that GTP may be causing a dissociation of SMP-III from its regulatory subunits. We examined the elution of SMP-III from a Superose 12 HR10/30 column in the presence and absence of GTP to determine whether differences in the molecular weight could be observed. The elution volume for the SMP-III phosphatase activity was identical in both cases suggesting that GTP does not cause SMP-III to dissociate.

GTP inhibits the activity of all the phosphatases tested. Since a type 1 like catalytic subunit is common to all phosphatases we hypothesized that GTP was interacting directly with the catalytic subunit, thereby causing a decrease in the phosphatase activity. In order to test our hypothesis, we performed a photoaffinity labelling of the different phosphatases. The different phosphatases were incubated with [α-³²P] GTP at room temperature. GTP was then cross-linked to the phosphatases by irradiating the mixture with ultraviolet light. Figure 4.44 shows a typical autoradiograph of SMP-III, SMP-IIIc and PP1δ after photoaffinity labeling. Several bands were labeled, however, none of these corresponds with the catalytic subunit. A single radioactive band is present in the SMP-IIIc lane at the interface of the running and stacking gels. This may be due to the aggregation of proteins present in the preparation. Since PP1δ showed no binding to GTP the aggregation observed with SMP-IIIc is unlikely to be the catalytic subunit itself. Myosin was also included in this study. A
Figure 4.43. Effect of pH on the inhibitory effect of GTP on the activity of SMP-III towards MLC$_{20}$ (A) and HMM (B) at pH 7.4 (□) and pH 7.0 (●).
strong radioactive label is observed for the heavy chains while a much weaker signal corresponds to MLC$_{20}$. These observations suggest that GTP may be affecting the phosphatase activity through its interaction with the substrate.

4.11 Effect of PK-C phosphorylation on the properties of SMP-III

Several studies have suggested that the state of smooth muscle contraction can be affected by activating PK-C with phorbol esters. It has been postulated that the activation of PK-C results in the inhibition of the myosin phosphatase leading to contraction of the smooth muscle. We have shown that the 130 and 22 kDa proteins that co-purify with SMP-III catalytic subunit are substrates for PK-C (figure 4.45). We examined the effect of this phosphorylation on the activity, inhibitor 2 sensitivity and myosin binding properties of SMP-III. ATP$_\gamma$S was used in the phosphorylation of SMP-III to prevent subsequent dephosphorylation. Table 4.9 shows the specific activity of SMP-III, with and without PK-C phosphorylation, towards MLC$_{20}$ and HMM. The activity of SMP-III was not significantly affected by PK-C phosphorylation.

The effect of PK-C phosphorylation on the sensitivity of SMP-III to inhibitor 2 was determined (figure 4.46). Phosphorylated and unphosphorylated SMP-III were incubated with inhibitor 2 and their activities towards MLC$_{20}$ and HMM was determined. Phosphorylation does not affect the sensitivity of SMP-III to inhibitor 2.
Figure 4.44 GTP photoaffinity labeling. SMP-III (a), SMP-IIIc (b), PP1δ (c) and myosin (d) were incubated with $[\alpha^{32}P]$ GTP at room temperature and then cross-linked, on ice, with low wavelength UV light. The samples were subjected to SDS PAGE and autoradiographed.
Figure 4.45 Protein kinase C phosphorylation of SMP-III. Lane a- SMP-III and PK-C, lane b- SMP-III alone, lane c- PK-C alone.
Table 4.9. Effect of protein kinase C phosphorylation on the activity of SMP-III towards MLC$_{20}$ and HMM

<table>
<thead>
<tr>
<th></th>
<th>MLC$_{20}$</th>
<th>HMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>12.1</td>
<td>2.3</td>
</tr>
<tr>
<td>PK-C phosphorylation</td>
<td>10.8</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Assay conditions: 50 mM Tris-HCL, 1 mM DTT, 0.8 µM MLC20, 0.4 µM HMM, 30 °C

The effect of PK-C phosphorylation of the myosin binding properties of SMP-III were also examined. Phosphorylated and unphosphorylated SMP-III were incubated with myosin. The mixture was centrifuged and the activity remaining in the supernatant was determined. A $K_{\text{binding}}$ of 1.4x10$^6$ M$^{-1}$ was obtained for both the control and the PK-C phosphorylated SMP-III suggesting that PK-C phosphorylation of these proteins is not involved in the regulation of SMP-III binding to myosin.
Figure 4.46. The effect of PK-C phosphorylation on the sensitivity of SMP-III to inhibitor 2. The phosphatase activities of unphosphorylated SMP-III (●), PK-C phosphorylated SMP-III (○), and PP1C' (□) were determined towards MLC$_{20}$ (A), HMM (B).
5.0 Discussion

The mechanism for the contraction and relaxation of smooth muscle depends on the reversible phosphorylation of MLC$_{20}$. The phosphorylation reaction, catalyzed by MLCK, has been well characterized. However, little is known about the dephosphorylation reaction or the myosin phosphatase that catalyzes this reaction. The main focus of this project was to purify and characterize a myosin phosphatase from turkey gizzards that may be involved in the *in vivo* dephosphorylation of myosin.

Previous studies with turkey gizzards have identified four phosphatases in the cytosolic fraction. These phosphatases have been termed smooth muscle phosphatase (SMP)-I, II, III, and IV. SMP-I is a heterotrimer composed of 60, 55, and 36 kDa subunits. This phosphatase is active towards MLC$_{20}$ but not towards intact myosin (Pato and Adelstein, 1983a). SMP-II is a Mg$^{2+}$ dependent monomeric phosphatase (43 kDa) which, like SMP-I, is active towards MLC$_{20}$ but not towards intact myosin (Pato and Adelstein, 1983b). The inability of these phosphatases to dephosphorylate intact myosin makes it unlikely that they are directly involved in the relaxation of smooth muscle. SMP-IV has also been purified to homogeneity from turkey gizzards (Pato and Kerc, 1985). This enzyme was originally reported as a dimer of 58 and 40 kDa subunits, however, western blot analysis comparing SMP-IV with recombinant PP1C shows that the catalytic subunit of SMP-IV has the same molecular weight as the recombinant phosphatases which is reported to be 38,000
(figure 4.26). Unlike SMP-I and II, SMP-IV is active towards intact myosin and, therefore, may play a direct role in the relaxation process. The fourth phosphatase found in smooth muscle cytosolic extracts is SMP-III. Crude preparations of this phosphatase are highly specific for intact myosin. The main focus of this project was the purification and characterization of this phosphatase.

5.1 Purification of SMP-III

The protocol for the purification of SMP-III is summarized in figure 4.12. Gel filtration of the turkey gizzard cytosolic extract on Sephacryl S-300 is a crucial step in the purification since it separates SMP-III from the other phosphatases. SMP-III (peak A) elutes shortly after the void volume and before SMP-I and IV (peak B) and SMP-II (peak C) (figure 4.2). This suggests that SMP-III has a molecular weight greater than 160 kDa, the molecular weight of SMP-I. Indeed, purified SMP-III elutes from a Sephadex G-200 gel filtration column calibrated with molecular weight standard proteins like a globular protein of 390 kDa.

Purification of SMP-III required several additional chromatographic steps. Heparin-Sepharose has been shown to separate type 1 and type 2 phosphatases (Gergely et al., 1984; Erdodi et al., 1985). Type 1 phosphatases bind well to this resin while type 2 phosphatases bind poorly to this resin. SMP-III binds to Heparin-Sepharose and is eluted at 0.4-0.5 mM KCl indicating that SMP-III has some physical similarities to type 1 phosphatases. SMP-III required further purification since SDS polyacrylamide gels of active fractions from this column show many protein bands.
Purification to near homogeneity was accomplished by affinity chromatography on thiophosphorylated HMM-Sepharose. SMP-III was chromatographed twice on thiophosphorylated HMM-Sepharose, first in the absence and then in the presence of 5 mM Mg$^{2+}$. Chromatography in the absence of Mg$^{2+}$ separated SMP-III from most of the contaminants. Addition of Mg$^{2+}$ to the equilibration buffer separated SMP-III from the remaining contaminants. These contaminants no longer bound to thiophosphorylated HMM-Sepharose in the presence of Mg$^{2+}$ suggesting that Mg$^{2+}$ reduces their affinity for the resin. Analysis of the eluate on SDS PAG showed three dominant bands at 130, 38, and 22 kDa (figure 4.13).

About 30 μg of SMP-III with a total activity of 13 nmol/min towards MLC$_{20}$ and 15 nmol/min towards HMM was obtained from 360 g of turkey gizzard smooth muscle. The fold purification for MLC$_{20}$ activity is 888 while the fold purification for HMM activity is 2505. The difference in these values is due to the separation of SMP-III from other protein phosphatases during the purification. Since two of the phosphatases (SMP-I and II) separated from SMP-III are active only towards MLC$_{20}$ the fold purification of the MLC$_{20}$ activity is less than that for HMM activity. Of interest is the apparent increase in total activity following (NH$_{4}$)$_{2}$SO$_{4}$ fractionation suggesting that the extract may contain a phosphatase inhibitor that does not precipitate at 30-60% (NH$_{4}$)$_{2}$SO$_{4}$.

5.2 Subunit structure of SMP-III

The SDS PAGE of SMP-III shows three prominent bands (figure 4.13). To determine whether these proteins are subunits of SMP-III the phosphatase preparation was subjected to non-denaturing polyacrylamide gel electrophoresis. Analysis of the active fractions eluted from the gel
showed that the 38 kDa protein correlates with activity (figure 4.19). Neither the 130 nor the 22 kDa were present suggesting that they are not subunits of SMP-III or that if they are, their binding to the catalytic subunit is weak.

To investigate the latter possibility attempts to immunoprecipitate the catalytic subunit with an anti-PP1C monoclonal antibody were made. These experiments showed that anti-PP1C monoclonal antibody does not immunoprecipitate the catalytic subunit well, thus, it cannot be visualized by coomassie or silver staining. Detection of the catalytic subunit is possible only by western blot analysis. Since the 130 and 22 kDa proteins are substrates of PK-C, their presence in the immunoprecipitated protein could be detected by phosphorylation with PK-C and [γ-32P] ATP. The autoradiograph following electrophoresis showed that proteins of 200, 130, and 68 kDa were enriched in the immunoprecipitate (figure 4.20b) suggesting that the 130 kDa may be a subunit of SMP-III since it is seen in the purified preparations. The relationship of the 200 and 68 kDa proteins which co-precipitate with the catalytic subunit but are not seen in purified SMP-III preparations is not clear at this time.

A number of myosin phosphatases with different subunit compositions have been purified from a variety of sources. Several different groups have reported the purification of myosin phosphatases from chicken gizzards. Onishi et al. (1982) have purified a myosin phosphatase with subunits of 67, 54, and 38 kDa. Alessi et al. (1992) have purified a heterotrimeric phosphatase (130, 37, and 20 kDa) termed smooth muscle PP1M from the myofibril fraction of chicken gizzards. MBP is a dimeric (58 and 38 kDa) myosin-associated phosphatase also purified from chicken gizzard (Okubo et al., 1993). Mitsui et al. (1992) have purified a
homotetramer of 34 kDa subunits termed MAPP that is also associated with the myofibrils of chicken gizzard. Turkey gizzards are another common source for smooth muscle phosphatases. Nowak et al. (1993) have purified a monomeric 35 kDa phosphatase from turkey gizzard myofibrils. SMP-IV is a dimer of 38 and 58 kDa subunits purified from the cytosol of turkey gizzard smooth muscle. Werth et al. (1982) have purified a dimer of 67 and 38 kDa subunits from bovine aorta. Two myosin phosphatases termed PT-1 and PT-2 have been purified from bovine heart (Mumby et al., 1987). PT-1 is a trimer of 63, 55, and 38 kDa subunits while PT-2 is a dimer of 63 and 38 kDa subunits. Dent et al. (1992) have purified a myosin bound phosphatase from rabbit skeletal muscle termed skeletal muscle PP1M which has a 33 kDa catalytic subunit.

The large number of myosin phosphatases described to date have quite different subunit compositions; however, some similarities do exist. The phosphatases purified by Onishi, Mumby, Werth and coworkers are similar to one another with subunits of 38 and 63-67 kDa. The dimeric subunit composition of SMP-IV and MBP is also similar. SMP-III and smooth muscle PP1M both appear as heterotrimers on SDS PAGE while MAPP and the myofibrillar phosphatase of Nowak appear as catalytic subunits of 34 and 35 kDa, respectively. The one common feature shared by all these phosphatases is a catalytic subunit with a molecular weight in the range of 33-38 kDa.

5.3 Classification of SMP-III

Ingebritsen and Cohen (1983) have proposed a scheme for the classification of serine/threonine phosphatases based on two criteria. The first criterion is based on the sensitivity of the phosphatase to heat stable
inhibitor 2. Type 1 phosphatases are inhibited while type 2 phosphatases are resistant to the inhibitor. SMP-III is not affected by inhibitor 2 suggesting that it is a type 2 phosphatase.

The second criterion is the dephosphorylation of the α or β subunit of phosphorylase kinase. Type 1 phosphatases preferentially dephosphorylate the β subunit while type 2 phosphatases preferentially dephosphorylate the α subunit. SMP-III is unable to dephosphorylate either subunit. In this respect SMP-III is unlike either type 1 or type 2 phosphatases.

However, SMP-III has some properties of type 1 phosphatases. First SMP-III binds tightly to Heparin-Sepharose which is a property common to type 1 phosphatases. Second, SMP-III is inhibited by okadaic acid at concentrations similar to those that inhibit type 1 phosphatases (figure 4.25). The sensitivity of phosphatases to okadaic acid is often used to determine the type of phosphatase involved in a particular cellular reaction (Cohen et al., 1989). Third, SMP-III cross-reacts with several type 1 specific antibodies but not with type 2A or type 2C antibodies suggesting structural similarities with type 1 phosphatase. Fourth, amino acid sequencing of proteolytic peptides shows that SMP-III has sequence similarities with a type 1 phosphatase isoform PP1δ (figure 4.28).

These properties suggest that SMP-III is a type 1 phosphatase but with respect to the main criteria for the classification, the activity towards phosphorylase kinase and sensitivity to inhibitor 2, SMP-III does not behave like a typical type 1 or type 2 phosphatase. SMP-III is not the only phosphatase that does not fit this classification scheme. Andres and Maller (1989) have described a ribosomal S6 phosphatase from Xenopus laevis that is also inactive towards phosphorylase kinase. A ribosomal S6
phosphatase has been also purified from rabbit reticulocytes that requires Mn\(^{2+}\) for activity and is sensitive to inhibitor 2 only when incubated with the inhibitor in the absence of Mn\(^{2+}\) (Wollny et al., 1984). Honkanen et al. (1991) have purified a phosphatase from bovine brain that is stimulated by inhibitor 2.

Several myosin-associated phosphatases have recently been described that do not fit the classification scheme. The rabbit skeletal muscle PP1M described by Dent et al. (1992) as well as the smooth muscle PP1M described by Alessi et al. (1992) are resistant to inhibitor 2 yet they dephosphorylate the \(\beta\) subunit of phosphorylase kinase. Both these phosphatases share amino acid sequence similarities with PP1\(\delta\) and have been classified as type 1 phosphatases. The uncharacteristic behavior of these phosphatases has been attributed to their regulatory subunits. The 37 kDa catalytic subunit of smooth muscle PP1M can be dissociated from the regulatory subunits by dilution in 4 M LiBr and gel filtration. Characterization of the isolated catalytic subunit revealed that it is sensitive to inhibitor 2 supporting its classification as a type 1 phosphatase and leading to the hypothesis that the regulatory subunit(s) shields the holoenzyme from inhibition by inhibitor 2. Similarly, the 33 kDa catalytic subunit of the skeletal muscle PP1M has been separated from its regulatory subunit resulting in a type 1 phosphatase-like sensitivity to inhibitor 2 (Dent et al., 1992).

In contrast to the PP1M phosphatases, the isolated catalytic subunit of SMP-III, SMP-IIIc, remains insensitive to inhibitor 2 suggesting that inhibitor 2 sensitivity is not an inherent property of all type 1 structurally-related phosphatases. Only after limited proteolysis were SMP-III and SMP-IIIc converted to inhibitor 2 sensitive phosphatases. Figure 4.30
shows that after 45 minutes of digestion SMP-III is mainly in a 34 kDa form similar to PP1C' but its activity is inhibited by inhibitor 2 to a lesser extent than the PP1C' control (figure 4.31). The sensitivity of proteolysed SMP-III to inhibitor 2 is not unlike that of MAPP which is also only partially inhibited by inhibitor 2 (Mitsui et al., 1992). It is interesting that 30 minutes of proteolysis converted SMP-IIIc into a phosphatase whose inhibitor 2 sensitivity is identical to that of PP1C' (figure 4.33) suggesting that regulatory subunit(s) are involved in moderating the effect of inhibitor 2.

Figure 4.30 shows that limited proteolysis converts the catalytic subunit of SMP-III from a 38 kDa protein, through a 37 kDa intermediate, to a final product of 34 kDa. After proteolysis for 45 minutes the majority of the phosphatase exists as a 34 kDa protein similar to PP1C'. Examination of the 34 kDa proteolytic products of other type 1 phosphatases shows that the N-terminal end remains acetylated indicating that proteolysis occurs at the C-terminus (Silberman et al., 1984; Flinta et al., 1986). The observations that the 34 kDa form of SMP-III is partially inhibited by inhibitor 2 while proteolized SMP-IIIc is inhibited to the same extent as PP1C' suggests that the C-terminal portion of the molecule is at least partly responsible for the resistance to inhibitor 2 and that the regulatory subunit(s) of SMP-III holoenzyme may be involved in protecting the phosphatase from inhibition by inhibitor 2.

Limited proteolysis of SMP-IV also results in an inhibitor 2 sensitive phosphatase. However, the extent of this sensitivity depends on the substrate used. The activity towards HMM is more strongly inhibited than activity towards MLC20 (figure 4.34). This difference may reflect the involvement of the regulatory subunit in enzyme activity and its
involvement in substrate binding. If the conformation of the 58 kDa regulatory subunit is altered by binding to HMM but not to MLC_{20} then this subunit may afford less protection from inhibitor 2 inhibition when HMM is used as substrate.

The second criterion for phosphatase classification is the dephosphorylation of phosphorylase kinase. SMP-III is unable to dephosphorylate phosphorylase kinase (figure 4.24). When first isolated, SMP-IV was reported to dephosphorylate the β subunit of phosphorylase kinase (Pato and Kerc, 1985). However, more recent preparations of SMP-IV did not dephosphorylate phosphorylase kinase (figure 4.35). Digestion of SMP-III and SMP-IV resulted in activation of these phosphatases to the β subunit of phosphorylase kinase (figure 4.35). Both SMP-III and SMP-IV become active towards phosphorylase kinase after 5 minutes of digestion. This corresponds to the formation of a 37 kDa species suggesting that the loss of a peptide of approximately 1 kDa from the C-terminus is sufficient to alter the phosphatase activity. The myosin-associated phosphatases reported by Alessi et al. (1992), Mitsui et al. (1992) and Dent et al. (1992) are all active towards the β subunit of phosphorylase kinase. The molecular weights of the catalytic subunits of these phosphatases are 37, 34 and 33 kDa, respectively. It is possible that these enzymes had undergone some limited proteolysis during purification which has resulted in their ability to dephosphorylate phosphorylase kinase.

A second possibility is that the catalytic subunits of these phosphatases differ from those of SMP-III and IV. Amino acid sequence analysis of proteolytic peptides from the catalytic subunits of skeletal and smooth muscle PP1M shows sequence similarities with PP1δ (Alessi et al., 1992; Dent et al., 1992). SMP-III also has sequence similarities to PP1δ.
(figure 4.28) suggesting that these phosphatases may have the same or similar catalytic subunits. However, since recombinant PP1δ is sensitive to inhibitor 2 while SMP-III is not, it is possible that these phosphatases have some structural differences. Also, it is important to consider the fact that PP1δ is expressed in *E. coli* where post translational modifications do not occur. It is therefore possible that expressed recombinant phosphatase has not undergone some post translational modification that maybe necessary for resistance to inhibitor 2. Another possibility is that the recombinant phosphatase is improperly folded. The recombinant phosphatases expressed in *E. coli* require Mn$^{2+}$ for activity (Zhang *et al.*, 1992). This suggests that the phosphatase have been improperly folded or processed since Mn$^{2+}$ is not required for the activity of purified type 1 phosphatases. Improper folding of PP1C expressed in *E. coli* has been suggested in a recent study whereby proper folding was induced by expressing the protein in the presence of inhibitor 2 which acts as a chaperone (Alessi *et al.*, 1993). In order to ascertain if SMP-IIIc and the catalytic subunits of smooth and skeletal muscle PP1M are indeed the same as PP1δ the cDNA for these phosphatases must be sequenced and compared.

### 5.4 Purification of SMP-III$\text{M}$

During the course of this project several studies were published describing the purification of myosin phosphatases from the myofibril fractions of chicken and turkey gizzards (Mitsui *et al.*, 1992; Alessi *et al.*, 1992; Okubo *et al.*, 1993; Nowak *et al.*, 1993). These studies prompted the examination of the myofibril pellet following the low salt cytosolic extraction. About 75% of the turkey gizzard smooth muscle myosin
phosphatase activity was found to be associated with the myofibril fraction. For this reason the purification of the myofibril-associated phosphatase was attempted.

A re-extraction of the myofibril pellet with 0.5 M KCl solubilized the myosin phosphatase activity. This phosphatase was purified in a similar manner to the purification of SMP-III. As seen with the cytosolic phosphatases, gel filtration chromatography fractionates the myofibril phosphatase activities into two peaks. The elution volumes of these peaks are nearly identical to those for peaks A (SMP-III) and B (SMP-I and IV) obtained in the purification of the cytosolic phosphatases (figure 4.2). Western blot analysis of these peaks show that they contain phosphatases that are structurally related to type 1 but not to type 2 phosphatases indicating that SMP-I is absent from the preparation. For these reasons we suspect that these phosphatases are myofibril-bound versions of SMP-III and SMP-IV.

Purification of the SMP-III_M peak was carried out. The phosphatase was purified to near homogeneity using Heparin-Sepharose and thiophosphorylated MLC_{20}-Sepharose chromatography. SDS PAGE analysis of the purified phosphatase shows three prominent bands at 130, 38, and 22 kDa (figure 4.18). Because of its structural similarities to SMP-III and its myofibril origin this phosphatase has been termed SMP-III_M. This protein is also structurally similar to smooth muscle PP1M from chicken gizzards (Alessi et al., 1992).

Classification of SMP-III_M was also attempted. As seen with SMP-III, SMP-III_M is not affected by inhibitor 2 and is unable to dephosphorylate phosphorylase kinase. SMP-III_M cross-reacts with anti-PP1 antibodies.
These observations further suggest that SMP-III\textsubscript{M} is a myofibril bound form of SMP-III.

5.5 Substrate specificity of SMP-III

SMP-III activity towards a variety of phosphoproteins has been determined. Table 4.2 shows that SMP-III has the highest activity towards myosin, HMM and MLC\textsubscript{20} and little or no activity towards phosphorylase \textit{a}, phosphorylase kinase or histone IIA. A similar study has compared SMP-III activity towards smooth muscle caldesmon and calponin with its activity towards MLC\textsubscript{20} (Pato \textit{et al.}, 1993). In this study SMP-III activity towards caldesmon phosphorylated by PK-C was about 35% of its MLC\textsubscript{20} activity while caldesmon phosphorylated with either cdc2 kinase or calcium-calmodulin dependent protein kinase II was very poorly dephosphorylated by SMP-III (2.0 and 1.7% of MLC\textsubscript{20}, respectively). Calponin phosphorylated by PK-C was dephosphorylated at about 50% the rate of MLC\textsubscript{20} dephosphorylation.

Other myosin phosphatases described in the literature have broader substrate specificity. Werth \textit{et al.} (1983) have described a dimeric phosphatase (38 and 67 kDa) active towards MLC\textsubscript{20} and intact myosin. However, unlike SMP-III, this phosphatase dephosphorylates histone IIA at about the same rate as MLC\textsubscript{20}. A MLC\textsubscript{20} phosphatase has been isolated from bovine aorta which dephosphorylates actomyosin as well as phosphorylase \textit{a} and therefore lacks the specificity of SMP-III (DiSalvo \textit{et al.}, 1983). The myosin associated phosphatase MAPP is highly specific for MLC\textsubscript{20} but has low activity towards caldesmon, histone III and phosphorylase kinase (Mitsui \textit{et al.}, 1992). Its activity towards intact myosin was not determined. Smooth muscle PP1M from chicken gizzards
showed similar activities towards both HMM and phosphorylase a (Alessi et al., 1992). The different substrate specificities may be due to differences in the subunit composition or the result of degradation of the phosphatases during purification. It is also possible that these phosphatases are different isoforms.

5.6 Kinetic properties

Both MLC$_{20}$ and HMM are good substrates for SMP-III with $K_M$ values of 2 and 5 $\mu$M, respectively, and $V_{max}$ values of 210 and 670 nmol/min/mg, respectively.

Proteolysis of SMP-III not only affects its activity towards phosphorylase kinase and its sensitivity to inhibitor 2 but also affects its activity towards MLC$_{20}$ and HMM. The MLC$_{20}$ and HMM phosphatase activities of SMP-III are stimulated by limited tryptic digestion (figure 4.29). Partial proteolysis has been previously reported to activate inactive phosphorylase phosphatase (Brautigan et al., 1982; Ballou et al., 1983). Chymotryptic digestion of PP1 to a 33 kDa species has been shown to increase the $K_M$ of the phosphatase for phosphorylase a, phosphorylase kinase and glycogen synthase. In contrast, we found that proteolysis to a 37 kDa species results in an increase in $V_{max}$ but no change in $K_M$. The differences observed in these studies may be explained by the different extents of proteolysis. However, these studies agree that the C-terminal portion of the phosphatase is important in modulating phosphatase activity. The role of the C-terminus in phosphatase activity is supported by a recent study which examined the effect of synthetic peptides corresponding to the C-terminus of PP1C on the activity of PP1C'+. (Martin et al., 1991). In this study a peptide containing a 5 amino acid sequence
found at the C-terminal region of PP1α stimulated PP1C' activity towards both phosphorylase a and MLC20. This sequence, with one conservative substitution (Ile to Val), is also present in the C-terminal region of all the other isoforms.

5.7 Characterization of SMP-III

5.7.0 Effect of divalent cations

Since divalent cations are known to regulate the activity of many enzymes the effect of divalent cations on different phosphatase activities has been studied many times in the past 20 years. The effects are often contradictory. Kato and Bishop (1972) have reported a stimulation of glycogen synthase phosphatase activity by Mn²⁺, Mg²⁺, and Ca²⁺. Brautigan et al. (1980) have reported that phosphorylase phosphatase activity is stimulated by Mn²⁺ but not affected by Mg²⁺ or Ca²⁺. Other studies have shown that phosphorylase phosphatase activity is inhibited by Mn²⁺, Mg²⁺, and Ca²⁺ (Brandt et al., 1975). It has been proposed that the differences in cation effect may be due to different substrates or impurities in the phosphatase preparations (Khandelwal, 1977).

The effect of divalent cations has also been determined for a number of smooth muscle phosphatases. The effects of divalent cations are as varied among these phosphatases as they were among the phosphatases described above. Werth et al. (1982) have purified a phosphatase from bovine aorta that requires Mn²⁺ or Co²⁺ for activity. Mg²⁺ or Ca²⁺ have no effect on activity of this phosphatase. The activity of MAPP from chicken gizzard is not affected by either Mg²⁺ or Ca²⁺-calmodulin (Mitsui et al., 1992). Okubo et al. (1993) have reported that the MLC₂₀ phosphatase activity of MBP is stimulated by Co²⁺ (max at 0.1 mM)
and Mn$^{2+}$ (max at 1-10 mM) but inhibited by Mg$^{2+}$ and Ca$^{2+}$ (at concentrations above 1 mM). Nowak et al. (1993) have described a monomeric 35 kDa phosphatase whose MLC$_{20}$ activity is stimulated by Mn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$ but is inhibited by Co$^{2+}$. The activity of SMP-IV from turkey gizzard is also affected by divalent cations (Pato and Kerc, 1985). Activity towards HMM is stimulated by both Mg$^{2+}$ and Ca$^{2+}$ while activity towards MLC$_{20}$ is inhibited by these cations.

Determination of the effect of several divalent cations on the activities of SMP-III and its isolated catalytic subunit SMP-IIIc towards HMM and MLC$_{20}$ showed similar effects on the activities of these enzymes. A divalent cation that stimulated (or inhibited) the phosphatase activities of SMP-III had the same effect on the activities of SMP-IIIc. The notable exception is the effect of Mg$^{2+}$ which inhibits SMP-III activity towards MLC$_{20}$ while it stimulates the activity of SMP-IIIc towards this substrate.

In contrast to the findings of Okubo et al. (1993) and Werth et al. (1982) but similar to those of Nowak et al. (1993) the activities of SMP-III and SMP-IIIc are potently inhibited by Co$^{2+}$. SMP-III activity towards MLC$_{20}$ and HMM is stimulated by Mn$^{2+}$ and in this respect SMP-III resembles the phosphatases described by Okubo, Nowak and co-workers.

At low concentrations of Mg$^{2+}$ (<10 mM) SMP-III activity towards HMM is stimulated while its activity towards MLC$_{20}$ is inhibited. However, Mg$^{2+}$ stimulates the activity of SMP-IIIc towards both these substrates. The effect of Mg$^{2+}$ on other myosin phosphatases is also variable. Werth and Mitsui and coworkers found that Mg$^{2+}$ had no effect on phosphatase activity. The MLC$_{20}$ activity of MBP from chicken gizzards is inhibited by Mg$^{2+}$ (Okubo et al., 1993). It is important to note
that the subunit structure of MBP closely resembles that of SMP-IV (dimer of 38 and 58 kDa) whose activity towards MLC_{20} is also inhibited by Mg^{2+}. In contrast, Nowak et al. (1993) reported that Mg^{2+} stimulates the MLC_{20} activity of a monomeric (35 kDa) turkey gizzard myosin bound phosphatase. This observation is consistent with the effect of Mg^{2+} on SMP-IIIc suggesting that the effect of Mg^{2+} may be influenced by the regulatory subunits when associated with the catalytic subunit.

5.7.1 Other effectors of SMP-III activity

The effect of ATP and NaF on phosphatase activity has been described in a number of studies. Many studies have reported the inhibition of phosphatase activity by ATP (Khandelwal, 1977; Ingebritsen et al., 1980, Khandelwal and Kamani, 1980, Mitsui et al., 1992). SMP-III is similar to these phosphatases in that it is also inhibited by ATP with IC_{50} values of 6 and 8 mM for MLC_{20} and HMM, respectively.

NaF is also a common inhibitor of phosphatase activity (Resink et al., 1983; Shacter-Noiman and Chock, 1983; Bollen and Stalmans, 1988, Mitsui et al., 1992). SMP-III is also inhibited by NaF and in this respect resembles other protein phosphatases.

5.8 Myosin binding studies

Myosin is a principal substrate for SMP-III in vitro suggesting a direct role for the phosphatase in the relaxation process. If indeed the physiological role of SMP-III is the dephosphorylation of myosin then it must also bind to myosin. Therefore, the examination of the binding of SMP-III to myosin should provide information regarding the regulation and localization of the phosphatase.
During the course of this study, Okubo et al. (1993) reported a $K_{\text{binding}}$ of $2.9 \times 10^6 \text{ M}^{-1}$ for MBP and demonstrated that the isolated catalytic subunit of MBP does not bind myosin. These authors suggested that the 58 kDa regulatory subunit of MBP is responsible for binding of the phosphatase to myosin. A subsequent study showed that the 58 kDa subunit has immunological similarities with a 130 kDa protein found in smooth muscle extracts leading to the speculation that the 130 kDa protein is the native form of the myosin binding subunit (Okubo et al., 1994). Contrary to this hypothesis, Nowak et al. (1993) reported a $K_{\text{binding}}$ of $5.45 \times 10^4 \text{ M}^{-1}$ for a monomeric 35 kDa myofibrillar phosphatase demonstrating that binding can occur in the absence of a regulatory subunit.

An earlier study by Sellers and Pato (1984) examined the myosin binding properties of impure SMP-III and SMP-IV. These authors reported $K_{\text{bindingS}}$ of 3.8 and $3.6 \times 10^5 \text{ M}^{-1}$ for SMP-III and SMP-IV, respectively. Our examination of the myosin binding properties of several purified turkey gizzard myosin phosphatases showed that SMP-III and SMP-III$_M$ bind to myosin with a $K_{\text{binding}}$ of $1.9 \times 10^6 \text{ M}^{-1}$ while SMP-IV has a slightly lower $K_{\text{binding}}$ of $1.1 \times 10^6 \text{ M}^{-1}$. The isolated catalytic subunit of SMP-III has the lowest $K_{\text{binding}}$ at $1.9 \times 10^5 \text{ M}^{-1}$. Although slightly different binding constants were found for the different myosin phosphatases all phosphatases tested bound strongly to myosin. These observations are contrary to the hypothesis that binding of myosin phosphatase to myosin is mediated by a specific myosin binding subunit.

Recombinant PP18 showed the highest affinity for myosin with a $K_{\text{binding}}$ of $5.1 \times 10^6 \text{ M}^{-1}$. Since PP18 was expressed in E coli its unusually strong binding may be due to the absence of post translational
modification(s) that may be important in binding to myosin. The anomalous binding may also result from improper folding of the phosphatase which is known to occur when these enzymes are expressed in a bacterial expression system (Alessi et al., 1993).

5.9 Regulation of myosin phosphatase activity
5.9.0 Effect of GTP on SMP-III

Recent studies have examined the effect of GTP and GTPγS on the contraction of permeabilized smooth muscle. GTPγS (100 μM) has been shown to stimulate tension development at submaximal calcium concentrations in permeabilized rabbit mesenteric artery (Nishimura et al., 1988; Fujiwara et al., 1989). A similar study using guinea pig ileum showed that the effect of GTPγS is inhibited by GDPβS suggesting the involvement of G-proteins. Subsequent studies have shown that the GTPγS-stimulated increase in tension correlates with an increase in MLC20 phosphorylation (Kitazawa et al., 1991a) at serine 19 (Kubota et al., 1992) suggesting either an activation of MLCK or an inhibition of the myosin phosphatase. Another study showed a decrease in the rate of MLC20 dephosphorylation which further suggests the regulation of the myosin phosphatase (Kitazawa et al., 1991b). In support of this hypothesis Kubota et al. (1992) showed that myosin light chain phosphatase activity is inhibited to 70 % of control in muscle homogenates from GTPγS (30 μM) treated tissue while MLCK activity is not affected. The general conclusion is that GTPγS activated G-protein signal transduction results in the inhibition of myosin phosphatase activity thereby increasing the calcium sensitivity. We examined the effect of GTP directly on the activity of SMP-III to determine if the increased calcium sensitivity may be due to direct
inhibition of the phosphatase activity by GTP. SMP-III activity is inhibited by GTP with an IC₅₀ of 400 µM for both MLC₂₀ and HMM at pH 7.4 (figure 4.43). When the pH is decreased to 7.0 the IC₅₀ decreases to 150 µM which is more in line with the above studies which were carried out at either pH 6.8 or 7.1.

We set out to determine the mechanism through which GTP was inhibiting the phosphatase activity. Our first hypothesis was that GTP induced a dissociation of the catalytic subunit of SMP-III from its regulatory subunit(s). This type of mechanism has been shown in the inhibition of myosin light chain phosphatase by arachidonic acid (Gong et al., 1992). GTP does not affect the elution of SMP-III from Superose 12 HR10/30 suggesting it does not promote dissociation.

A second possible mechanism is that GTP inhibits phosphatase activity through direct association with the enzyme. Since GTP inhibits SMP-III, SMP-IIIₘ, SMP-IIIc, SMP-IV, and recombinant phosphatases we proposed that GTP inhibition was mediated through direct association with the catalytic subunit. However, GTP photoaffinity labeling (figure 4.44) showed that GTP does not bind to the catalytic subunit. GTP does bind strongly to the myosin heavy chains; however, since GTP inhibits phosphatase activity equally towards MLC₂₀ and HMM it is unlikely that this represent the mechanism of inhibition. A lesser degree of binding was observed for the 20,000 Da light chains which may be involved in the inhibitory action of GTP.

A third possibility is that GTP affects phosphatase activity by affecting the affinity of the phosphatase for its substrate. This hypothesis was tested by determining the Kₘbinding of SMP-III and SMP-IIIc to myosin in the presence and absence of GTP (figure 4.42). The Kₘbinding for SMP-III
was increased from $1.9 \times 10^6 \ M^{-1}$ to $2.6 \times 10^6 \ M^{-1}$ while that of SMP-IIIc was increased from $1.9 \times 10^5 \ M^{-1}$ to $7.7 \times 10^5 \ M^{-1}$. An increase in binding affinity may result in a decrease in phosphatase activity by slowing the movement of the phosphatase from one substrate molecule to the next.

5.9.1 Effect of PK-C phosphorylation on SMP-III

Several studies have suggested that protein kinase C activation produces contraction in smooth muscle (Chatterjee and Tejada, 1987; Rasmussen et al., 1987). Activation of PK-C in swine carotid artery and rabbit thoracic artery has been correlated with an increase in MLC$_{20}$ phosphorylation (Singer, 1990b; Seto et al., 1990). These effects can be inhibited by specific PK-C inhibitors (Brozovich et al., 1990). It was subsequently shown that MLC$_{20}$ phosphorylation occurred at serine 19, the site of MLCK phosphorylation (Itoh et al., 1993; Masuo et al., 1994). This observation suggests that PK-C does not affect contraction through the direct phosphorylation of MLC$_{20}$ but rather through the phosphorylation of some other protein(s) thereby modulating the level of MLCK catalyzed MLC$_{20}$ phosphorylation. Itoh et al. (1993) showed that myosin phosphatase activity was inhibited in muscle homogenates from phorbol ester treated tissue suggesting that the PK-C stimulated contraction is mediated through the inhibition of the myosin phosphatase. This hypothesis was reinforced by Masuo et al. (1994) who showed that when myosin phosphatase activity is inhibited by microcystin LR, phorbol esters produce no further change in the contractile state of the smooth muscle.

We have shown that the 130 and 22 kDa proteins that copurify with SMP-III are substrates for PK-C (figure 4.45). We hypothesized that SMP-III
activity may be directly affected by this phosphorylation, however, no change in activity was observed (table 4.9).

A second possibility is that PK-C phosphorylation of SMP-III might change its sensitivity to inhibitor 2 and thus subject it to inhibitor 2 regulation. However, figure 4.46 shows no difference in the inhibitor 2 sensitivity of either the phosphorylated or unphosphorylated SMP-III.

A third possible mechanism for regulating the dephosphorylation of myosin in vivo is the dissociation of the phosphatase from its substrate. This type of mechanism has been proposed for the regulation of PP1G. Phosphorylation of the PP1G glycogen binding subunit by PK-A promotes the translocation of the phosphatase from glycogen to the cytosol (Hiraga and Cohen, 1986; Hubbard and Cohen, 1989). Regulation of PP1G binding to glycogen by insulin-dependent protein kinase has also been reported (Dent et al., 1990). No change in the binding affinity of SMP-III for myosin was observed as a result of PK-C phosphorylation. Since neither PK-A nor MLCK phosphorylate SMP-III these enzymes are not likely to affect the myosin binding and thus the localization of SMP-III. It is possible that other kinases could phosphorylate SMP-III and thereby regulate its localization to the myofibrils.

5.10 Conclusions and speculations

Substrate specificity, kinetic and myosin binding studies suggest that the physiological role for SMP-III is the dephosphorylation of myosin. The myosin-binding studies and the immunological and enzymatic comparisons of SMP-III with SMP-III\textsubscript{M} suggests that these are cytoplasmic and myofibrillar forms of the same phosphatase. Comparison of the HMM phosphatase activity present in the cytosolic and myofibrillar
extracts suggests that most of SMP-III is bound to the myofibril. The presence of the phosphatase in the cytosol may be due to the extraction process or may be a result of the localization of the myosin phosphatase to specific subcellular locations as the result of some regulatory mechanism.

Comparison of SMP-III and SMP-IIIc show some differences in their enzymatic and myosin binding properties. These differences may be due to the presence of regulatory subunit(s) in the SMP-III preparation. Indeed SMP-III elutes from a calibrated gel filtration like a globular protein of 390,000 Da supporting the possibility of multiple subunits. Although immunoprecipitation experiments were not conclusive they do show that a 130 kDa protein coprecipitates with the catalytic subunit. In order to best explain these observations we hypothesize that SMP-III is a multisubunit enzyme most likely composed of a 38 kDa catalytic subunit and a 130 kDa regulatory subunit. It is possible that other subunits of SMP-III also exist.

Enzymatic, immunological, and proteolytic studies of SMP-III and SMP-IV suggest that these phosphatases share a common catalytic subunit. The few differences in their properties that have been observed may be due to their different subunit composition. The 58 kDa subunit of SMP-IV may be a proteolytic product of the 130 kDa subunit of SMP-III. This hypothesis is supported by a recent study showing immunological similarities between the 58 kDa subunit of MBP and a 130 kDa protein found in smooth muscle extracts (Okubo et al., 1994).

SMP-III, SMP-IIIc and SMP-IV have immunological similarities with type 1 phosphatases. Furthermore, amino acid sequencing of proteolytic peptides from SMP-III show similarities with PP1δ. However, these enzymes differ from the typical type 1 phosphatase in their sensitivity to inhibitor 2 and their activity towards phosphorylase kinase.
Only after limited proteolysis do these enzymes show type 1 phosphatase like enzymatic characteristics. The change in enzymatic properties correlates with the digestion of the C-terminal end of the phosphatases. We suggest that the catalytic subunits of these phosphatase contain a unique sequence at their C terminus that is responsible for their unique properties.

Numerous physiological and biochemical studies have suggested that the modulation of smooth muscle calcium sensitivity involves the regulation of myosin phosphatase activity. It has been suggested that the activation of PK-C results in a decrease in myosin phosphatase activity. We have explored the possibility that SMP-III activity is directly regulated by PK-C phosphorylation; however, no evidence for direct regulation of SMP-III activity by PK-C phosphorylation was found. This does not preclude the possibility of indirect regulation of SMP-III by PK-C. Further investigation of the effect of PK-C on myosin phosphatase activity is required.

A second group of studies have focused on the effect of GTP on smooth muscle calcium sensitivity. These studies suggest that GTP regulates myosin phosphatase activity through interaction with G proteins. We determined that SMP-III activity is directly affected by GTP at concentrations similar to those used in these studies. It is possible that myosin phosphatase activity is directly regulated by GTP and that G proteins are not involved in this process.
6.0 Summary

SMP-III is a smooth muscle phosphatase highly specific for myosin and myosin derivatives. All non-contractile proteins tested were poor substrates for SMP-III. This high degree of specificity suggests that SMP-III may be directly involved in the dephosphorylation of myosin in vivo and, therefore, involved in the relaxation process.

SMP-III has been purified to near homogeneity from the cytosolic fraction of turkey gizzard smooth muscle. The catalytic subunit of SMP-III has a molecular weight of 38,000 as determined by non-denaturing PAGE and western blot analysis. However, SMP-III elutes from a calibrated gel filtration column like a globular protein of 390,000 Da. This suggests that SMP-III is either a multisubunit enzyme or highly asymmetric. SMP-III co-purifies with proteins of 130 and 22 kDa; however, these proteins separate from the catalytic subunit under non-denaturing electrophoresis suggesting a weak association. Since some of the enzymatic properties of SMP-III and SMP-IIIc differ it is likely that regulatory subunits do exist. The exact nature of these subunits has yet to be determined.

The kinetic properties of SMP-III towards MLC$_{20}$ and HMM were determined. SMP-III has a $K_M$ of 2 and 5 $\mu$M for MLC$_{20}$ and HMM, respectively, while the $V_{\text{max}}$ is 210 and 670 nmol/min/mg for these substrates.

SMP-III activity is stimulated by limited tryptic digestion. Western blot analysis shows that digestion occurs at the C-terminus of the catalytic subunit. A 1.5- and 2.3-fold increase in the $V_{\text{max}}$ towards MLC$_{20}$ and
HMM, respectively, correlates with the cleavage of a peptide of approximately 1 kDa from the C-terminus of the phosphatase. No change in the $K_M$ was observed as a result of proteolysis.

As isolated, SMP-III cannot be classified as either a type 1 or type 2 phosphatase. SMP-III is insensitive to heat stable inhibitor 2 suggesting a type 2 phosphatase. However, SMP-III does not dephosphorylate either the $\alpha$ or $\beta$ subunits of phosphorylase kinase and is, therefore, unlike either type 1 or type 2 phosphatases. Western blot analysis indicates that SMP-III has structural similarities with type 1 phosphatases but not with either type 2A or type 2C phosphatases. Furthermore, amino acid sequencing of proteolytic peptides from SMP-III show similarities with PP1$\delta$. Limited tryptic digestion of SMP-III results in sensitivity to heat stable inhibitor 2 and activity towards the $\beta$ subunit of phosphorylase kinase, properties of type 1 phosphatases. The changes in enzymatic properties correlate with the proteolysis of SMP-III at its C-terminus. We have considered two possibilities to explain these observations. First, SMP-III may be a type 1 related phosphatase with a unique sequence at its C-terminus that results in altered enzymatic properties. Second, SMP-III catalytic subunit is PP1$\delta$ and the sensitivity to inhibitor 2 and activity towards phosphorylase kinase are not properties inherent to PP1$\delta$. To determine if SMP-III is in fact PP1$\delta$ it must be first cloned and sequenced.

The effect of a variety of reagents on SMP-III activity was determined. With respect to the effects of nucleotides, NaF and KCl, SMP-III behaves in a similar fashion to many other protein phosphatases. The effects of divalent cations is less uniform. Most myosin phosphatases described in the literature differ with respect to the effects of divalent
cations. The reason for these differences is unclear but may be related to subunit composition, purity, or degradation during purification.

SMP-III binds strongly to myosin with an $K_{\text{binding}}$ of $1.9 \times 10^6$ M$^{-1}$. Similar $K_{\text{binding}}$ values were observed for other myosin phosphatases examined including SMP-III$M$ ($K_{\text{binding}}$ of $1.9 \times 10^6$ M$^{-1}$), SMP-IV ($K_{\text{binding}}$ of $1.1 \times 10^6$ M$^{-1}$), and SMP-III$c$ ($K_{\text{binding}}$ of $1.9 \times 10^5$ M$^{-1}$). The strong binding of these phosphatases to myosin suggests that a specific myosin binding subunit is not required.

The regulation of SMP-III activity was examined. SMP-III is inhibited by GTP with an IC$_{50}$ of 400 μM at pH 7.4 and an IC$_{50}$ of 150 μM at pH 7.0. These values are in line with the concentrations of GTP and GTPγS used in permeabilized smooth muscle experiments showing an increase in Ca$^{2+}$ sensitivity in response to these nucleotides. These observations suggest that regulation of myosin phosphatase activity may be important in the regulation of the contractile state of smooth muscle.

The activation of PK-C in permeabilized smooth muscle results in an increased Ca$^{2+}$ sensitivity. It has been suggested that this effect is mediated by the inhibition of the myosin phosphatase. The effects of PK-C phosphorylation on several properties of SMP-III were examined. PK-C phosphorylation has no effect on SMP-III activity or its sensitivity to inhibitor 2. No effect on the binding of PK-C to myosin as a result of PK-C phosphorylation was determined. Since SMP-III is not directly affected by PK-C phosphorylation other processes are likely involved in the regulation of the myosin phosphatase activity. This mechanism of regulation requires further study.
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