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THE UNIVERSITY OF ALBERTA

Purification and Characterization of Two Novel Proteins (SM21 and SM22) from Smooth Muscle

by

James Paul Lees-Miller

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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OF DOCTOR OF PHILOSOPHY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Purification and Characterization of Two Novel Proteins (SM21 and SM22) from Smooth Muscle submitted by James Paul Lees-Miller in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Abstract

We have purified and characterized two novel proteins (SM21 and SM22) of low M_r from smooth muscle. SM21 was identified on denaturing polyacrylamide gels by its ability to bind ^{125}I-labeled tropomyosin. It is present in chicken gizzard smooth muscle at a ratio to actin of ≈ 1 : 16. SM21 was purified using both denaturing and non-denaturing techniques. Amino acid analyses indicated that SM21 has a high half-cystine and glycine content, and automated amino acid sequencing indicated that it was not similar to any previously sequenced protein. Physical characterization of non-denatured SM21 demonstrated that it is a moderately asymmetrical (globular) monomer, with a Stokes radius of 23.1 Å, a M_w of 21,200 and a sedimentation constant of 2.03 S. Calculations based on its far ultraviolet CD spectrum provided values of 17 % α-helix, 32 % β-sheet, 14 % β-turn, and 37 % random coil. SM21 was found to bind both calmodulin and actin using the gel overlay technique.

Chicken gizzard smooth muscle contains a highly abundant protein (SM22) with an apparent M_r on sodium dodecyl sulfate polyacrylamide electrophoretic gels of 23,000. The ratio of actin: SM22: tropomyosin in this tissue is estimated to be 6.5 (±0.8): 2.0 (±0.2): 1.0. At least three isoelectric isoforms are present in ratios of α:β:γ of 14:5:1 with α the most basic and γ the most acidic. Amino acid analyses of purified α and β demonstrate the presence of one and two half-cystines respectively and a lower

content of basic amino acids in β. A value of 22,000 for the M_w of α, estimated by sedimentation equilibrium indicated its presence as a monomer at physiological ionic strengths. Estimates of the translational frictional coefficient (f/f_{min}) of α calculated from its Stokes radius (25.5 Å) and M_w were consistent with its existence as a moderately asymmetric globular protein. Calculations based on its far ultraviolet CD spectrum provided values of 37% α-helix, 31% β-sheet, 5% β-turn and 27% random coil.

Using a rabbit polyclonal antibody preparation directed against the chicken gizzard protein we have demonstrated by immunoblotting the presence of SM22 in a variety of chicken smooth muscle containing organs including uterus, intestine, gizzard, esophagus and aorta. SM22 was present in only trace amounts in brain, liver and heart and could not be detected in chicken breast muscle. The antibody preparation did not cross react with SM22 in extracts of bovine aorta. However the presence of SM22 as a major component in bovine aorta and porcine carotid was demonstrated by its comigration with the purified chicken gizzard protein on one and two dimensional polyacrylamide electrophoretic gels. Its molar abundance relative to actin was estimated to be $\simeq$ 0.9 : 6 and 1.4 : 6 for bovine aorta and porcine carotid respectively. Purification of the aorta SM22 has shown it to have a similar amino acid composition to the chicken gizzard protein.

Acknowledgements

During the course of this study, several individuals have performed critical experiments pertaining to the purification and characterization of SM21 and SM22. As I have found it necessary to incorporate most of their work into this thesis, I would like to identify these people and the work which they carried out in whole or in part. Mike Carpenter ; automated sequencing, Krystyna Golosinska ; purification of gizzard SM22, David Heeley ; two-dimensional gels and phosphorylation studies, Vic Ledsham ; ultracentrifuge operation, Mike Nattriss ; amino acid analyses, Kim Oikawa ; CD spectroscopy, Joyce Pearlstone ; sequence of SM22, Clive Sanders ; purification of aorta SM21, Donald Stewart ; antibodies to SM22, Marion Weber ; sequence of SM22. I would also like to say that I received large amounts of useful advice, both technical and theoretical, from all of the above mentioned people.

I would like to thank Dr. Lawrence B. Smillie for (a) supervising my thesis project, (b) always taking an interest in my progress and (c) providing many of the important ideas upon which this work was based. I would also like to thank Dr. Cyril M. Kay for his generous help, both in the use of equipment for physical studies and in the interpretation of results from these studies. A thank you also goes to Susan and John for providing moral support throughout the course of this work.

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ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphatase
ATPγS	adenosine 5'-O(3'-thiotriphosphate)
CD	circular dichroism
CM-	carboxymethyl
CTP	cytidine 5'-triphosphate
cyclic AMP	adenosine 3',5'-cyclic phosphate
cyclic GMP	guanosine 3',5'-cyclic phosphate
DEAE-	diethylaminoethyl
DTT	dithiothreitol
[redacted]A	ethylenediaminetetraacetic acid
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
f/f_{min}	experimental translational frictional ratio
f/f_o	translational frictional ratio due to shape alone
F-actin	filamentous actin
G-actin	globular actin
HMM	heavy meromyosin
KMED	analytical buffer consisting of 0.1 M KCl, 50 mM 4-morpholinepropanesulfonic acid, 1 mM EGTA, 1 mM DTT, pH 7.0

LMM	light meromyosin
MLC	myosin light chain
MLCK	myosin light chain kinase
M_r	molecular weight
M_w	weight average molecular weight from sedimentation equilibrium
NMR	nuclear magnetic resonance
NTPase	nucleotide triphosphatase
R_s'	experimental Stokes radius
$R_{s,gel}$ ($R_{s,sed}$)	experimental Stokes radius determined by filtration (sedimentation velocity)
R_o	molecular radius of an equivalent sphere identical molecular weight and $\bar{v}$
$S_{20,w}$	sedimentation coefficient corrected to wa at 20°C
$S^*_{20,w}$	intrinsic sedimentation coefficient
S	Svedberg unit of sedimentation velocity (10^{-13} s)
S1	subfragment 1 of myosin
SDS-PAGE	sodium dodecyl sulfate-polyacryamide gel electrophoresis
T2	chymotryptic fragment of troponin T (resi 159-259)
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet

$\bar{v}$	partial specific volume
Vh	volt hour
w	protein hydration
ρ	gel filtration partition coefficient

I. Introduction

The generation of directional movement is an important function for most living organisms. The various means used to generate movement include cilia, flagella, cytoplasmic streaming, gel sol transformation, and contraction. During the development of multicellular animals contractile tissue (muscle) developed as the primary means of locomotion. It is widely distributed from the primitive cnidarians and acoelomate flatworms to the highly specialized vertebrates and arthropods. Muscle is broadly characterized into three types based on microscopic anatomy. These include the obliquely striated muscles of nematodes, annelids and molluscs, cross striated muscle in molluscs, arthropods and vertebrates, and smooth muscle in molluscs, echinoderms, lower chordates and in vertebrate internal organs. The phylogenetic relationship between the various muscle types is obscure.

Functionally, cross striated muscle is usually associated with rapid body movements, as best illustrated by the skeletal muscles of vertebrates and arthropods. It can also however be used for slow movement as in the alimentary canal of arthropods and the contractile ring of jellyfish (Hoyle, 1983). Obliquely striated, and smooth muscles are generally associated with slow movements. Some bivalve molluscs possess adductor muscles that combine a cross or obliquely striated portion that is used for shell closing, with a smooth portion that maintains tension on the closed

shell. In the case of scallops the cross striated portion is also used for propulsive movements (reviewed in Elliot and Bennett, 1982).

Our present understanding of how muscles function relies primarily on information obtained from the study of cross striated muscle. There are several reasons why this is the case. Vertebrate striated muscles are large and contain a relatively small amount of non-muscle tissue making them suitable for biochemical studies. They also have a highly ordered structure and are thus suitable for experiments in morphology using both microscopy and x-ray diffraction. These attributes are also useful for physiological studies. The combined effort from these fields of muscle research has led to a detailed understanding of the structure of cross striated muscle and its mechanism of contraction. For this reason I will briefly review our present knowledge of the striated muscle contractile machinery, and discuss the development of protein purification methods for this tissue, many of which have been adapted for use in this study. An excellent general review on the development of muscle research can be found in Needham (1971). A more up to date treatise on muscle has been written by Squire (1981).

A. Skeletal Muscle

Vertebrate skeletal muscle fibers are long cylindrical multinucleate cells. The fibers contain bundles of parallel myofibrils. Each myofibril is made up of a series of contractile units called sarcomeres (Fig. 1A). Each sarcomere contains a band of thick filaments that are anisotropic when viewed with polarized light (A band). The thick filaments consist of a bundle of myosin molecules arranged in a bipolar fashion (Fig. 1B). Each myosin molecule contains six subunits. Two of the subunits are heavy chains (Mr 200,000) which have globular heads and interact with each other through an elongated region of α-helical coiled coil known as the rod. Attached to each head are two myosin light chains (MLC), one of which can be removed by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB light chain) (Mr 19,000) and the other by alkali (Mr 15,000-25,000). The myosin heavy chains are particularly sensitive to proteolysis in two regions of the coiled coil. Proteolysis at one site produces heavy meromyosin (HMM) containing both heads and light meromyosin (LMM) containing the remainder of the rod. Cleavage at the second site removes individual heads, called subfragment-1 (S1), from the rod portion of the molecule (Fig. 1C).

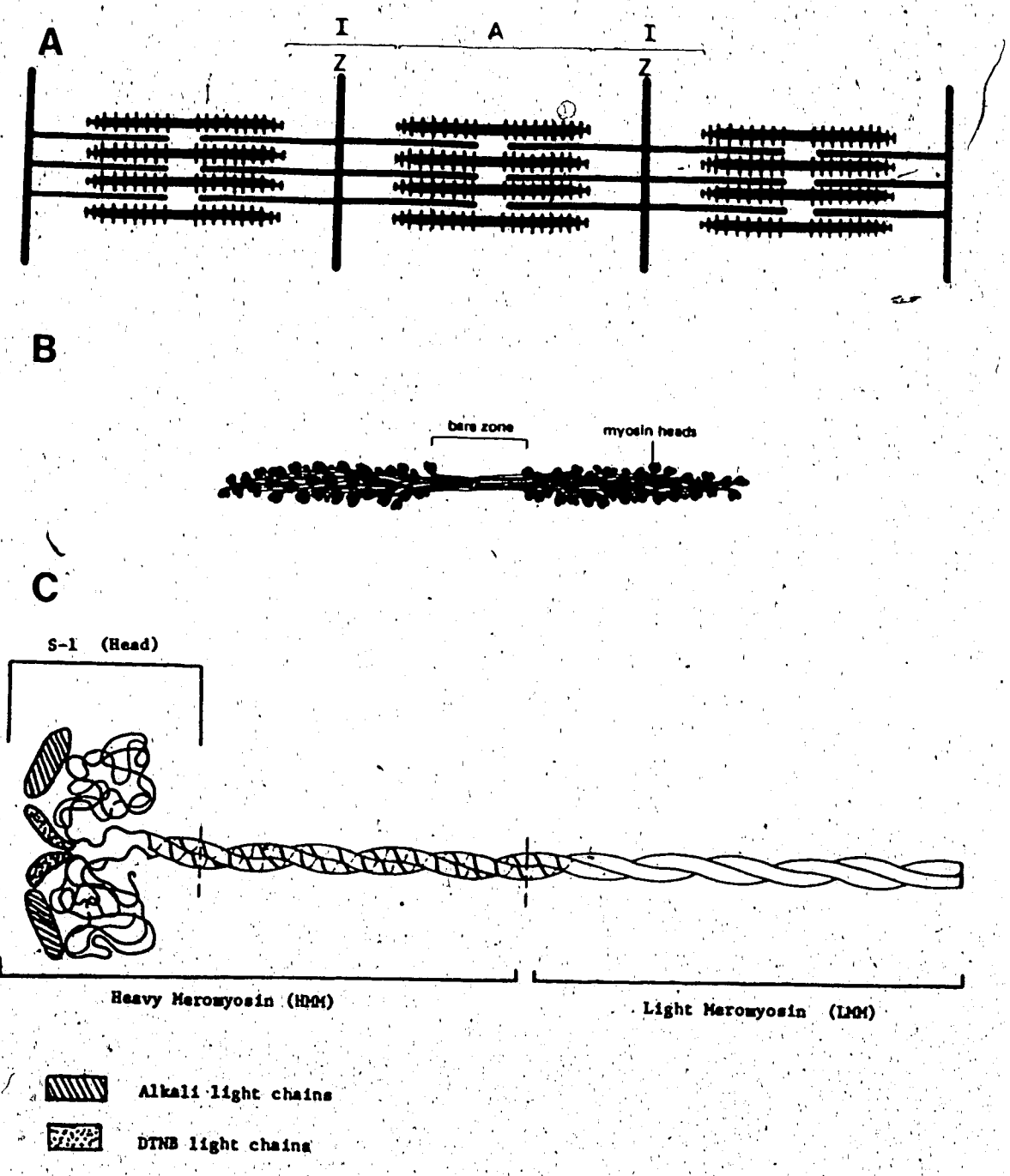

FIG. 1. Schematic representations of (A) the sarcomere [adapted from Huxley, 1969] (B) the thick filament [from Alberts et al., 1983] and (C) myosin.

Each sarcomere also contains an I band that is isotropic and consists of thin filaments that project from a dense Z line and interdigitate with the thick filaments in the A band (Fig. 1A). Thin filaments are made up of three proteins named actin, tropomyosin and troponin (Fig. 2). The filament core consists of globular (G) actin monomers (M_r 42,000) that have been polymerized into a double helical filament known as F-actin. A second filament, consisting of tropomyosin (M_r 66,000) polymerized in a head to tail manner, is coiled around each chain of the actin double helix. There is one tropomyosin for each 7 actin monomers. Attached to each molecule of tropomyosin there is one elongated molecule of troponin (M_r 70,000) which contains 3 subunits named I, T, and C. The amino acid sequences of the thin filament proteins have been determined (reviewed in Leavis and Gergely, 1984).

Interaction between thick and thin filaments occurs through binding of crossbridges, made up of the HMM portion of myosin, to actin. The force for sliding of filaments is provided by changes in the nature of the crossbridge attachment, referred to as crossbridge cycling. Energy to drive crossbridge cycling comes from the splitting of one ATP to ADP + Pi per crossbridge, for each cycle. Troponin and tropomyosin control the interaction of actin with the myosin crossbridge. In resting muscle they inhibit actomyosin ATPase activity while in activated muscle they potentiate it.

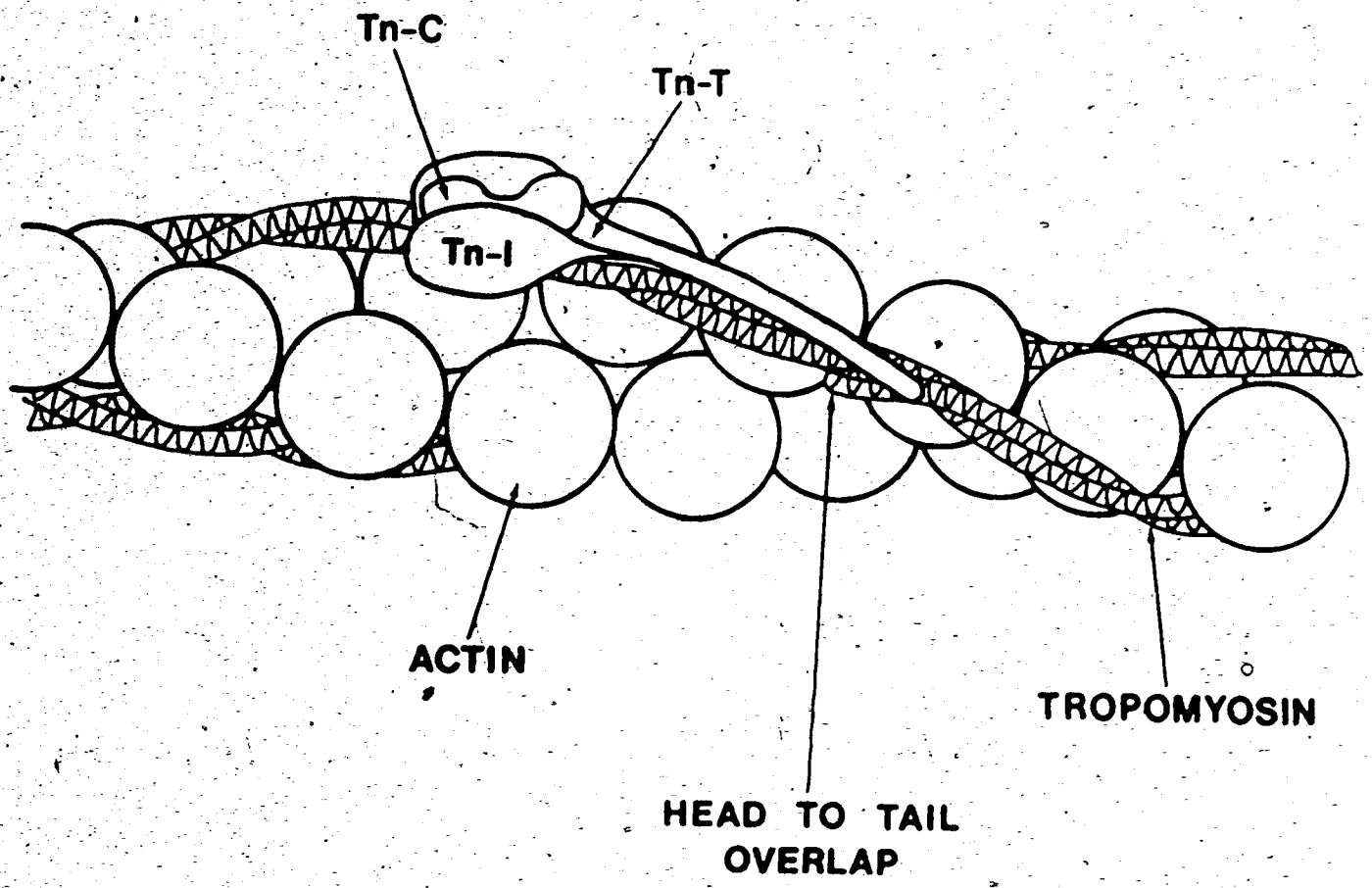

Fig. 2. Current model for the structure of the striated muscle thin filament. Abbreviations are Tn-C, troponin C; Tn-I, troponin-I, TnT, troponin T, [with permission from D.H. Heeley and L.B. Smillie].

Purification of Muscle Proteins

The first extractions of muscle proteins were made in 1859 when Kühne found that the syrupy liquid expressed during thawing of a frozen muscle powder would form a white precipitate on addition to cold water. He named the precipitate myosin (cited in Needham, 1971). Several other scientists including O. von Furth, W.D. Halliburton and H.H. Weber improved upon Kühne's preparation. Their work culminated in 1930 with J.T. Edsall's purification of a muscle globulin, now known to consist primarily of actomyosin. Edsall (1930) extracted fresh rabbit muscle with a solution containing 1.2 M KCl and 0.05 M K_2HPO_4 pH 7.0-8.5. The muscle globulin could then be precipitated by adding more salt to the extract or by diluting it with water. After several rounds of precipitation and resolubilization, a monodisperse solution of protein was obtained. Weber and Edsall improved on the purification of "myosin" during the 1930's, but it was the discovery in 1939 that ATP breakdown in muscle was associated with myosin (Engelhardt and Lyubimova, 1939) and the discovery of actin by Szent-Györgyi's group (reviewed by Szent-Györgyi, 1945 and 1951), which sparked the meteoric rise of muscle protein chemistry in the decades to come. In 1941 Szent-Györgyi noticed that a short extraction with alkaline Webers solution containing 0.6 M KCl led to a less viscous myosin (myosin A) than did a long extraction (myosin B). Straub treated the residue left after extraction of myosin A with

acetone. Extraction of the resulting powder with water yielded a new protein, which on addition to myosin A formed synthetic myosin B, now better known as synthetic actomyosin. The new protein was named actin. The extraction of myosin A was modified by Guba and Straub. They used a solution containing 0.3 M KCl, 0.15 M potassium phosphate pH 6.5. This solution is still used in modern day preparations, with the addition of ATP to reduce the interaction of actin and myosin (Margossian and Lowey 1982).

Another important protein discovered in the 1940s was tropomyosin (Bailey 1948). Tropomyosin purification also involves production of an acetone powder which is followed by high salt extraction and isoelectric precipitation at pH 4.6. The similar structural characteristics of myosin and tropomyosin (both contain extended helical rods) led to the idea that tropomyosin might be a precursor of myosin. Later studies demonstrated that tropomyosin is an independent component of the thin filament.

In 1952 Hasselbach was able to demonstrate that actin could activate the myosin ATPase activity, at low salt (0.1 M KCl), in the presence of Mg^{2+}. This activation was not seen in the presence of high salt indicating the importance of the interaction between actin and myosin. Thus by the early 1950s a basic understanding of the actin-myosin-ATP interaction had been elucidated, but no one physical mechanism of contraction could be agreed upon.

The sliding filament mechanism of contraction was put forward primarily by H.E. Huxley and A.F. Huxley during the 1950s (reviewed by Huxley, 1960). The original theory was based on structural studies using both microscopy and X-ray diffraction to observe changes in the relationship of thick and thin filaments during contraction. The selective extraction of myosin from myofibrils allowed the assignment of myosin to the anisotropic band (thick filaments) and actin to the isotropic band (thin filaments) (Hanson and Huxley, 1953). Two important aspects that the early sliding filament theory did not address were the mechanism by which the actomyosin ATPase was regulated and the role of tropomyosin in muscle. It was later realized that these two problems were related.

By the 1950s it was evident that calcium was important in the regulation of muscle contraction (reviewed by Huxley 1980). Marsh (1952) isolated a Ca^{2+} dependent relaxing factor from muscle now better known as the sarcoplasmic reticulum. Using citrate to control Ca^{2+} levels, Bozler (1954) demonstrated that glycerinated muscle required Ca^{2+} for contraction. Perry and Grey (1956) used ATP and EDTA to control levels of divalent cations, and found that natural actomyosin was Ca^{2+} sensitive whereas synthetic actomyosin was not. Other evidence indicated that some synthetic actomyosins were Ca^{2+} sensitive while others were not, depending on the method of actin preparation (Weber and Winicur, 1961). Ebashi (1963) finally put all the clues

together and tried to isolate a more natural actin by eliminating acetone treatment prior to extraction according to Straub. He found that the protein resulting from this extract was more like tropomyosin than actin and that in the presence of Ca^{2+} it would activate natural actomyosin, which had been previously desensitized to Ca^{2+} by treatment with trypsin. Ebashi and Kodama (1965) were able to separate natural tropomyosin into two components by precipitating out a Bailey-like tropomyosin at pH 4.6. The resulting supernatant had low viscosity but contained a protein that greatly increased the viscosity of tropomyosin and also resensitized trypsin treated natural actomyosin to Ca^{2+}. Ebashi named the new protein troponin.

The discovery of troponin and its interaction with tropomyosin helped to consolidate the growing evidence that tropomyosin was located along with actin on the thin filament. Perry and Corsi (1958) used selective extraction with low salt to demonstrate that actin and tropomyosin were both located in the I band of myofibrils. Electron microscope studies indicated the presence of a 40 nm repeat within the I band (Carlsen *et al.*, 1961). This repeat was absent in images of F-actin which led Hanson and Lowy (1963) to suggest that it was due to tropomyosin wrapped around the actin filament. Laki *et al.* (1962) noted that attempts to purify actin by inducing a G to F transition with 0.1 M KCl left actin with about 25 % tropomyosin contamination. They then prepared tropomyosin free G-actin. When tropomyosin was

added to this actin followed by polymerization and centrifugation, it was found to make up 19 % of the resulting F-actin pellet. This is a close approximation to the presently accepted 1:7 molar ratio of tropomyosin to actin. Thus there was strong evidence that tropomyosin made up part of the actin filament.

Troponin was shown to be present on the thin filament by Ohtsuki (1967). He used antibodies against troponin to demonstrate its presence at 40 nm intervals in the I band of myofibrils. Lehman *et al.* (1972) isolated native thin filaments from skeletal muscle and confirmed that they consisted primarily of actin, tropomyosin and troponin.

The final step in elucidating the components of the thin filament was the identification of troponin's subunits. This work was greatly aided by the introduction of sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) (Shapiro *et al.*, 1967), which also helped to identify the contaminants present in previous muscle protein preparations and led to an improved understanding of the subunit composition of all the major muscle proteins. Greaser and Gergely (1971) purified troponin by following Bailey's procedure for isolating tropomyosin. After precipitation of tropomyosin at pH 4.6, troponin was recovered from the supernatant and separated into three components by ion exchange chromatography in the presence of 8 M urea. SDS-PAGE was then used to identify the apparent M_r and purity of each component. Greaser and Gergely (1973)

assigned functions to each of the components. Troponin C (M_r — 18,000-20,000) was found to bind Ca^{2+}, Troponin I (M_r 22,000-24,000) contained most of the actomyosin inhibitory activity and troponin T (M_r 37,000) was found to interact with tropomyosin. It should be noted that other groups led by D.J. Hartshorne, S.V. Perry and S. Ebashi were simultaneously coming to similar conclusions. By the early 1970s the concept of myosin crossbridge cycling had also been developed. Thus a good overall understanding of skeletal muscle structure,function and regulation had been obtained. Stated briefly, the cytoplasm of resting muscle contains 0.1 - 0.5 μM free Ca^{2+} and 1 - 2 mM free Mg^{2+}. The free Mg^{2+} inhibits myosin ATPase activity in the absence of an appropriate interaction with actin. This interaction is prevented by the troponin-tropomyosin complex in the presence of low Ca^{2+} levels. The muscle is activated by a nervous stimulus causing membrane depolarization. The depolarization results in release of Ca^{2+} from the sarcoplasmic reticulum which elevates cytoplasmic Ca^{2+} to the 1 - 5 μM range. More Ca^{2+} then binds to troponin C causing a conformational change that alters the interaction of troponin I - troponin T - tropomyosin complex with actin, allowing it to activate myosin ATPase. The resulting release of energy is used to alter the interaction of the myosin crossbridge with actin in a manner that results in a net displacement of actin and myosin filaments relative to each other. The displacement is transmitted to the skeleton by a

series of connective tissue elements, resulting in movement. Relaxation is accomplished by removing Ca^{2+} through a Ca^{2+} pump in the sarcoplasmic reticulum.

Studies since the early 1970s have been aimed at refining this fundamental knowledge to the level of molecular and atomic interactions. Some examples include (A) determination of the structure of troponin C at high resolution (Herzberg and James, 1985), and the structure of S1-actin (Amos *et al.*, 1982) and tropomyosin (Phillips *et al.*, 1986) at low resolution, (B) structure and regulation of the thin filament (reviewed in Leavis and Gergely, 1984), (C) actin-HMM interaction and kinetics (Chalovich and Eisenberg, 1986), (D) time resolved X-ray diffraction studies of contractile movements within intact muscle (Kress *et al.*, 1986), and (E) isolation of cDNAs and genes coding for the contractile proteins (for a recent summary see Emerson *et al.*, 1986).

B. Smooth Muscle

Smooth muscle can be generally defined as a tissue that is specialized for contraction but does not show obvious sarcomeric organization with standard light or electron microscope techniques. Several invertebrates possess smooth skeletal muscles. The smooth adductor muscle of bivalve molluscs can be used to hold their valves tightly closed, with a minimal expenditure of energy. This state is referred to as catch. In this type of muscle an asymmetric protein called paramyosin is present in the core of myosin filaments. It is believed to be involved in the catch mechanism (reviewed in Achazi (1982). Regulation of contraction in catch muscle occurs primarily through binding of Ca^{2+} to a regulatory light chain of myosin (Ashiba *et al.*, 1980). Smooth muscle is present in most deuterostome invertebrates including brachiopods, echinoderms, and lower chordates (reviewed in Hoyle, 1983).Of particular interest is the smooth body wall musculature of ascidians (protochordata) which is known to possess thin filament linked regulation and troponin (Toyota *et al.*, 1979). There is however no compelling evidence to indicate that the body wall muscle of ascidians has a closer evolutionary relationship to vertebrate visceral muscle which is smooth than to vertebrate body wall muscle which is striated.

The main concern of this thesis is vertebrate smooth muscle. This tissue type is located in many organs, including blood vessels, trachea, urinary bladder and ducts,

genital ducts, digestive tract, and eye. Smooth muscle is usually found in layers which can be orientated longitudinally, circumferentially,or randomly depending on the organ type. An exception to this rule is the avian gizzard which possesses thick lobes of smooth muscle and is thus a choice tissue for initial biochemical characterization.

Physiology

Smooth muscle innervation comes primarily from the autonomic nervous system and is not subject to voluntary control. Most smooth muscles exibit two types of contractile response, termed phasic and tonic. The phasic response is associated with an action potential, while tonus usually occurs as a summation of phasic contractions. Phasic and tonic contractions in smooth muscle are similar to twitch and tetanus in skeletal muscle. Smooth muscle contracts slowly relative to striated twitch muscle. For example, gastric muscle was found to have a latent period of 0.77 s, a rise time of 2.2 s and a half relaxation time of 2.8 s (McSwiney and Robson, 1929). This can be compared to the slow twitch soleus muscle which completes the entire process in about 0.2 s.

Smooth muscle cells are mononucleate. Cell to cell communication occurs through regions of apposed membrane that have low electrical resistance (Abe and Tomita, 1968). This type of communication is important in both smooth and

cardiac muscle for transmission of electrical impulses between cells. An intercellular junction known as the gap junction has been correlated with the presence of low intercellular resistance, in virtually all tissues that possess it. Smooth muscle is the one possible exception to this rule in that some smooth muscles are found to lack clearly defined gap junctions while retaining low resistance communication (Daniel *et al.*, 1983). Many smooth muscles do however have gap junctions.

Diversity in vertebrate smooth muscle arises primarily from differences in the means of excitation and the factors that modify excitability. For example the taenia coli undergoes spontaneous contraction, and contracts in response to stretch, but receives little innervation. The vas deferens is well innervated but does not contract spontaneously or in response to stretch. In both cases however, the stimulus is propagated by an action potential and low resistance cell to cell junctions. There exists a cornucopia of nervous and hormonal factors that can modify smooth muscle contractility. These include catecholamines, acetylcholine, histamine, prostaglandins, ATP, and a long list of peptides including oxytocin, angiotensin, enkephalin, and atrial natriuretic factor (reviewed in Bolton, 1979, and Burnstock, 1985). The effect of each factor can differ depending on the muscle involved. In most cases substances produce an effect on smooth muscle by altering the membrane potential. This is referred to as

electromechanical coupling, as opposed to pharmacomechanical coupling where a response is elicited without a change in membrane potential (Casteels, 1980).

Coupling of stimulus and response in smooth muscle requires changes in cytoplasmic Ca^{2+} levels. The Ca^{2+} neccessary to induce a response can enter the cytoplasm either through the plasma membrane or from the endoplasmic reticulum. Morphological studies using electron probe analysis have demonstrated that the endoplasmic reticulum contains enough Ca^{2+} to elicit a full response and that it does release more than enough Ca^{2+} to account for contraction following a stimulus (Somlyo, 1985). Earlier work (Brading *et al.*, 1969) had shown that Ca^{2+} rather than Na^{+} carries most of the inward current during a smooth muscle action potential. However, Johansson and Somlyo (1980) determined that the amount of Ca^{2+} entering a cell during an action potential was insufficient to account for the increase in cytoplasmic Ca^{2+}. Thus the mechanism by which plasma membrane depolarization causes Ca^{2+} release from the endoplasmic reticulum is an important issue in smooth muscle studies, as it is in skeletal muscle. A recent suggestion is that the chemical transmitter inositol 1,4,5, triphosphate (reviewed in Berridge and Irvine, 1984) which is a breakdown product of phosphatidyl inositol may play a role in Ca^{2+} release. Cyclic AMP and cyclic GMP have also been shown to play a role in smooth muscle excitation contraction coupling (reviewed in Kroeger, 1983). The task

of sorting out the interactions between Ca^{2+}, inositol 1,4,5, triphosphate, cyclic AMP and cyclic GMP dependent second messenger systems is presently receiving considerable attention.

Structure

Smooth muscle cells are spindle shaped with a centrally located nucleus. In standard histological preparations their cytoplasm appears amorphous. Isolated smooth muscle cells do however demonstrate several properties indicative of fibrillar organization. Small (1974) used both polarizing optics and phase contrast to demonstrate fibrils in skinned cells and Fisher and Bagby (1977) found that the birefringence properties of isolated stomach cells were altered with contraction. Cultured smooth muscle cells were also found to have a fibrillar organization when stained with antimyosin antibodies (Gröschel-Stewart *et al.*, 1975).

With the higher resolution of electron microscopy, smooth muscle cells are seen to contain 3 main filament systems. These are (1) actin-tropomyosin containing thin filaments, 6-8 nm in diameter, (2) desmin and vimentin containing intermediate filaments, 10 nm in diameter and (3) myosin containing thick filaments, 14-16 nm in diameter. Smooth muscle also contains dense plaques associated with membranes and dense bodies distributed throughout the cytoplasm. These electron opaque structures have been shown to anchor both thin and intermediate filaments (reviewed in

Bagby, 1983). Bond and Somlyo (1982) used negative staining of cells treated first with saponin and then with myosin S1 to demonstrate that actin filaments leaving dense bodies in opposite directions have opposite polarity. These structures may therefor be analogous to the Z lines of skeletal muscle. High voltage electron microscopic images of thick sections, viewed in stereo have revealed cross bridges between thick and thin filaments (Ashton *et al.*, 1975). More recently these crossbridges have been detected in freeze-fracture deep-etch studies which provide an excellent 3 dimensional image (Somlyo and Franzini-Armstrong, 1985). Taken together these studies indicate that contraction in smooth muscle occurs by a sliding filament mechanism. Compared to skeletal muscle ,the major difference lies in the way myofibrils are organized.

Metabolism

Metabolism in smooth muscle appears to be essentially similar to that found in other better characterized tissues such as skeletal muscle and liver. It does however have some interesting properties associated with contractile function. Contraction in rat portal vein is followed by a significant increase in oxygen consumption, but does not increase lactate production (Hellstrand 1977). The only change in phosphagen content that occurred during contraction was a drop in phosphocreatine. Other researchers have found high aerobic lactate production in smooth muscle. Several lines

of evidence indicate that the ATP made during lactate production is used predominantly for ion transport across membranes (reviewed in Lundholme *et al.*, 1983). These findings suggest that separate energy compartments exist, one using oxidative phosphorylation to support contraction and the other glycolysis for membrane transport. However Tomita *et al.*, (1985) have found that hydroxybutyrate, which can only be used in oxidative phosphorylation will support membrane pumping activity. A better understanding of metabolism in smooth muscle might be obtained through purification and characterization of the control enzymes. Little progress has been made in this direction.

Actomyosin

Early studies of smooth muscle actomyosin ATPase followed in the footsteps of those done on skeletal muscle (reviewed by Needham, 1971). Some unique and important properties were however discovered. Laszt and Hamoir (1961) found that actomyosin could be extracted from bovine carotid with a low salt solution (ionic strength 0.05 M) that contained traces of ATP. This discovery reflects the relative instability of the thick and thin filaments of smooth muscle, and has been capitalized on in modern purification techniques. When Bárány *et al.* (1966) purified myosin from chicken gizzards, they found that it possessed ATPase activity in the presence of either Ca^{2+} or EDTA. The Mg^{2+} ATPase was however not activated by actin. Although not

realized at the time, these findings were an indication that smooth muscle myosin must be activated prior to becoming sensitive to actin.

A native tropomyosin was isolated from chicken gizzard by Ebashi *et al.* (1966). They found that it could restore Ca^{2+} sensitivity to the superprecipitation of synthetic actomyosin. A Ca^{2+} sensitizing factor was also extracted from a natural arterial actomyosin using low salt (Sparrow and van Bockxmeer, 1972). The components of this fraction were analysed by SDS-PAGE and found to consist mainly of tropomyosin with a lesser amount of troponin-like components. Carsten (1971) prepared a troponin-like protein from an acetone powder of bovine uterus. On SDS-PAGE the major bands were located at 26 and 15 kDa, and minor bands in the 43-70 kDa range were also identified. These studies appeared to be establishing a role for troponin in the regulation of smooth muscle contraction. At about the same time however evidence was accumulating for a myosin linked regulatory system in vertebrate skeletal muscle and molluscan muscle.

Myosin Linked Regulation

Kendrick-Jones *et al.* (1970) found that myosin from either striated or smooth scallop adductor muscle was Ca^{2+} sensitive in the presence of Mg^{2+} and purified (unregulated) actin. The Ca^{2+} sensitivity of scallop myosin was found to reside in its M_r 19,000 light chains (Szent-Györgi *et al.*,

1973). These results represented the first demonstration of myosin linked regulation.

Phosphorylation of the Mr 18,500 light chain of skeletal muscle myosin was demonstrated by Perrie *et al.* (1973). They found that the light chains migrated as two bands on an alkaline urea gel and that the only difference between the bands was a single phosphate. The presence of the phosphate group on myosin did not affect its ATPase activity. It would however turn out to have more profound effects on smooth muscle myosin.

Sobieszek and Bremel (1975) purified a Ca++ sensitive actomyosin from chicken gizzard that possessed a considerably higher ATPase activity than previous preparations. The first step was to prepare myofibrils by carrying out low salt extractions in the presence of Triton X-100, which helped to remove membranes and membrane proteins. Actomyosin was then extracted from the myofibrils with a low salt solution containing 10 mM ATP and 1 mM EDTA. It was shown not to contain troponin-like subunits by SDS-PAGE. Bremel (1974) used this preparation to demonstrate that addition of unregulated actin to Ca^{2+} sensitive actomyosin does not affect Ca^{2+} regulation providing additional evidence against thin filament linked regulation. Phosphorylation of smooth muscle myosin was demonstrated by Sobieszek (1977). He found that addition of Ca^{2+} to smooth muscle actomyosin resulted in phosphorylation of the 20 kDa MLC. This phosphorylation was followed by an activation of

actomyosin ATPase activity.

The next step was to identify the kinase responsible for the phosphorylation of myosin. Gorecka *et al.* (1976) extracted a fraction from a Ca^{2+} sensitive actomyosin that could phosphorylate the 20 kDa MLC in a Ca^{2+} sensitive manner. Partial purification of myosin light chain kinase (MLCK) was also achieved by Sobieszek and Small (1977). During this study they discovered that MLCK could be isolated in greatest quantity by extracting myofibrils with a solution containing 15-30 mM Mg^{2+}. This solution has been adapted for most modern MLCK preparations (Walsh *et al.*, 1983 ; Sobieszek and Barylko, 1984). Dabrowska *et al.* (1978) demonstrated that MLCK consists of two subunits. One had a high M_r and possessed the catalytic activity. The other had a low M_r, bound Ca^{2+} and was identical to calmodulin. Calmodulin is a ubiquitous Ca^{2+} binding protein that in addition to activating MLCK also mediates the Ca^{2+} sensitivity of some forms of cyclic nucleotide phosphodiesterase, adenylate cyclase, phosphorylase kinase, and Ca^{2+}-Mg^{2+} ATPase as well as being involved in many important physiological processes (reviewed in Walsh and Hartshorne, 1983).

The development of knowledge on MLCK led to a hypothesis for regulation of contraction in smooth muscle (reviewed in Adelstein and Eisenberg, 1980). Stated briefly, a stimulus, nervous, hormonal or mechanical causes an increase in intracellular Ca^{2+} concentration. The excess

Ca^{2+} binds to calmodulin which then binds to and activates MLCK. Active MLCK phosphorylates the regulatory light chains of myosin resulting in active actomyosin ATPase, crossbridge cycling and net filament sliding. Relaxation results from removal of Ca^{2+} by the endoplasmic reticulum and dephosphorylation of MLC by a MLC phosphatase (Pato and Kerc, 1985).

The myosin linked regulatory system can be modulated in several ways. MLCK is a substrate for cAMP dependent protein kinase. The phosphorylation of MLCK reduces its affinity for calmodulin resulting in a decreased ability to phosphorylate myosin in the presence of Ca^{2+} (Conti and Adelstein, 1981). Substances that relax smooth muscle by increasing cAMP levels may exert their influence through this pathway. An example is adrenergic relaxation (Stull *et al.*, 1983). However, Miller *et al.* (1983) found that beta-adrenergic stimulation of tracheal smooth muscle did not alter the level of calmodulin sensitivity of MLCK in this tissue, arguing against MLCK phosphorylation as a physiological mechanism of smooth muscle relaxation.

Early studies demonstrated that fully phosphorylated actomyosin from chicken gizzard was not sensitive to Ca^{2+} concentration (Sobieszek, 1977), while that from guinea pig vas deferens was (Chacko *et al.*, 1977). However, Seidel *et al.*, (1986) found that phosphorylated actomyosin ATPase from both bovine artery and gizzard are stimulated 3-4 fold by a solution containing 0.2 mM Ca^{2+} versus one containing

EGTA. Ikebe and Hartshorne (1985b) have found that Ca^{2+} only has a significant effect on actomyosin ATPase activity when Mg^{2+} levels are low (0.1 mM) making the significance of Ca^{2+} activation uncertain at the present time.

Substances that affect the conformation of smooth muscle myosin also affect its ATPase activity. Suzuki *et al.* (1978) found that unphosphorylated myosin filaments in a solution containing 0.15 M KCl and 10 mM $MgCl_2$ were disassembled by addition of ATP. The resulting myosin sedimented rapidly at 10 S and was converted to a slower sedimenting 6 S form on addition of KCl to 0.3 M. The slower sedimenting form had an increased ATPase activity. Under the electron microscope, the 10 S form appears to fold back on itself near the HMM junction (Trybus *et al.*, 1982) accounting for its greater sedimentation rate. Phosphorylation of myosin favors the 6 S form. However, under low salt conditions which favor 10 S formation, phosphorylation does not increase the ATPase or cause the 10 S to 6 S transition (Ikebe *et al.*, 1983). The important point is that phosphorylation increases ATPase by favoring the 6S conformation, so that any other factor that affects the transition can theoretically inhibit or potentiate the ATPase. Partial proteolysis of 6 S myosin with *Staphylococcus aureus* protease results in formation of both HMM and S1. Similar treatment of 10 S myosin produces only HMM (Ikebe and Hartshorne, 1985). HMM is activated by actin in a phosphorylation dependent manner similar to that of

whole myosin. S1 is also activated by actin but in this case phosphorylation has no effect. The subfragment-2 region which lies between the S1 and HMM cleavage sites is therefor thought to coordinate the effect of MLC phosphorylation on actomyosin ATPase activity. Knowledge that MLC phosphorylation is cooperative and that phosphorylation of both S1 heads is required for full ATPase activation (Persechini and Hartshorne, 1981) also supports this idea, although other studies (Trybus and Lowey, 1985) have indicated that MLC phosphorylation is random.

Contraction in Skinned Fibers and Whole Muscle

Work with skinned smooth muscle fibers has tended to provide strong support for the myosin phosphorylation theory (reviewed by Kerrick *et al.*, 1984). Contraction of skinned fibers is Ca^{2+} dependent and is associated with MLC phosphorylation. Adenosine 5'-O(3'-thiotriphosphate), (ATPγS) acts as a substrate for MLCK but the resulting thiophosphorylated MLC can not be dephosphorylated by MLC phosphatase. It therefor causes irreversible phosphorylation of MLC in the presence of MLCK and Ca^{2+}. The presence of ATPγS, ATP and Ca^{2+}in skinned fibers causes an activation of contraction that is not reversed when Ca^{2+} is removed, providing strong support for the role of phosphorylation in smooth muscle contraction (Hoar *et al.*, 1979). Addition of extra calmodulin to skinned fibers reduces the amount of Ca^{2+} required for contraction and calmodulin inhibitors

block both MLC phosphorylation and contraction in accordance with their relative abilities to inhibit calmodulin dependent enzyme activity. Conclusive evidence that MLC phosphorylation is sufficient for development of tension was provided by Walsh *et al.* (1982) through the use of a chymotryptic fragment of MLCK which retains kinase activity but is not Ca^{2+} sensitive. They found that this kinase increased both tension and MLC phosphorylation in the absence of Ca^{2+}.

The early studies on whole muscle preparations also tended to support the phosphorylation theory (reviewed in Asano and Stull, 1985). The general correlation of tension and MLC phosphorylation was first questioned by Driska *et al.* (1981). Using strips of carotid artery, they discovered that MLC phosphorylation increased with a high K^+ stimulus, and this increase preceded force development in keeping with skinned fiber studies. They also found that MLC phosphorylation decreased prior to maximal tension being developed and that tension was maintained after dephosphorylation was complete. In smooth muscle the velocity of contraction (crossbridge cycling rate) can be varied independently of force (number of crossbridges attached)(Dillon *et al.*, 1982). MLC phosphorylation correlates with velocity but not force in smooth muscle strip preparations. These findings led Aksoy *et al.* (1982) to postulate the existence of a second regulatory mechanism that is activated following dephosphorylation of myosin and

exerts its effect by strengthening crossbridge attachment. These slow cycling crossbridges are referred to as latch bridges.

When smooth muscle fibers are activated a transient increase in Ca^{2+} occurs that is similar to the MLC phosphorylation transient and correlates with tension development. It is followed by a plateau in Ca^{2+} levels that correlates with gradual relaxation (Morgan and Morgan, 1982). Aksoy *et al.* (1983) found that myosin phosphorylation was less sensitive to external Ca^{2+} concentration than was stress maintenance. They concluded that the factor controlling stress maintenance was regulated at lower Ca^{2+} levels than MLC phosphorylation. Skinned fibers were also shown to maintain tension at Ca^{2+} levels too low to support MLC phosphorylation (Chatterjee and Murphy, 1983).

Hoar *et al.* (1985) demonstrated that addition of MLC phosphatase to skinned fibers that were submaximally stimulated by Ca^{2+} resulted in an increase in tension. The same phosphatase decreased tension of fibers activated by Ca^{2+} insensitive MLCK in the absence of Ca^{2+}. In both cases, whether tension increased or decreased, the level of phosphorylated MLC was reduced by phosphatase treatment. These findings confirmed the presence of a second Ca^{2+} dependent mechanism for force production or maintenence in smooth muscle that depends on phosphorylation-dephosphorylation for its activation. The presence of this second factor helps to explain why MLC phosphorylation

levels in skinned and whole fibers rarely exceed the 20-50 % range while isolated actomyosin requires 100 % phosphorylation for its full activation. Indeed, the second regulatory system is predicted to be capable of producing a slow rise in tension even when myosin phosphate is cycling on and off with no net increase. CTP can support the actomyosin NTPase but not myosin phosphorylation. Skinned smooth muscle cells do not contract in the presence of CTP and Ca^{2+}, showing that the second system requires prior phosphorylation of MLC for its activation. If the fibers are first treated with ATP they can maintain tension in the presence of CTP, Ca^{2+} and phosphatase (Hoar *et al.*, 1985). This appears to be an ideal system for testing the effect of potential regulatory elements on tension maintenance. Several factors have been described that might fulfill a regulatory role in smooth muscle.

Thin Filament Linked Regulation

Leiotonin

When Ebashi's group attempted to isolate a thin filament linked regulatory system from smooth muscle they did not find troponin but did find a protein which they called leiotonin. This protein consists of two subunits ; one 80 kDa subunit and one 17 kDa Ca^{2+} binding subunit. The 17 kDa subunit differs from calmodulin and troponin C in amino acid composition and electrophoretic mobility. Calmodulin can functionally

replace it in the leiotonin complex, but it does not activate calmodulin dependent phosphodiesterase (reviewed in Nonomura and Ebashi, 1980). Leiotonin can activate superprecipitation of actomyosin or its ATPase activity in a ratio of less than 1:50 with actin. It does not require a high level of myosin phosphorylation for its activity but does require tropomyosin, actin and Ca^{2+} (reviewed in Ebashi *et al.*, 1982). Leiotonin is thin filament linked (Mikawa, 1979). Problems with the leiotonin system include the lack of complete purification and an explanation of how it could act at such a low ratio to actin.

Tropomyosin

Smooth muscle tropomyosin activates actomyosin ATPase when the ATP and salt concentration, and the molar ratio of myosin heads to actin are in the physiological range (reviewed in Marston and Smith, 1985). Under similar conditions skeletal tropomyosin is inhibitory, but it can also be activating if ATP is low and myosin:actin ratio is high (Bremel *et al.*, 1972). The ability of high myosin occupation on actin to induce skeletal tropomyosin from an inhibitory state into an activating state indicates that it can alter the interaction of tropomyosin with actin. It would therefor be expected that tropomyosin could alter the binding of myosin to actin. Williams *et al.* (1984) demonstrated that smooth muscle tropomyosin favors the activating

position on actin even at low S1 concentration as long as the ionic strength is high enough (0.12 M). It also favors the binding of S1 to actin at lower S1 concentrations than does skeletal tropomyosin. The significance of these results is not yet clear, but if a factor exists in smooth muscle that can alter the state of tropomyosin on actin, it will also be capable of affecting S1 binding and possibly the maintenance of tension.

Caldesmon

Caldesmon is another smooth muscle protein that appears to be thin filament linked and might be involved in regulation of contraction. It has an apparent M_r on Laemmli gels of 140,000. Caldesmon binds to and can bundle actin filaments (Bretscher, 1984). In the presence of Ca^{2+}, calmodulin binds to caldesmon and reduces its affinity for actin (Sobue *et al.*, 1981). Sobue *et al.* (1982) found that caldesmon could inhibit superprecipitation of actomyosin that was phosphorylated by MLCK and contained tropomyosin. The inhibition was only Ca^{2+} dependent if calmodulin was present in a 1:1 molar ratio with caldesmon. Caldesmon can also inhibit actomyosin ATPase activity, but a 1:1 calmodulin:caldesmon ratio does not reverse the inhibition (Ngai and Walsh, 1984). The inhibition of actomyosin ATPase activity is greatest when tropomyosin is present but also occurs in its absence. Ngai and

Walsh (1984) also purified caldesmon that had been phosphorylated in a Ca^{2+}-calmodulin dependent manner and found that it did not inhibit actomyosin ATPase. They proposed that phosphorylation of caldesmon might regulate its inhibitory effect.

Caldesmon is known to be present in some Ca^{2+} sensitive thin filament preparations (Marston and Smith, 1985) and has been localized on actin containing filaments in non-muscle cells by immunofluorescence (Bretscher and Lynch, 1985). Lash *et al*. (1986) have found that caldesmon can increase the affinity of actin for myosin and proposed that it could be involved in tension maintenance. Some problems with assigning a role for caldesmon in regulation include (1) a mechanism for turning it on and off that is reproducible by more than one group and (2) an explanation of how it can function at an intracellular ratio of about 1:40 with actin. Further complicating factors include the presence of caldesmon in skeletal muscle (Ngai and Walsh, 1985a), its ability to inhibit myosin ATPase in the absence of actin (Lim and Walsh, 1986) and its actin filament bundling activity. Recently, Szpacenko and Dabrowska (1986) isolated a proteolytic fragment of caldesmon, with an M_r of 18,000, that binds both actin and calmodulin. Any amino acid sequence similarity between this fragment and troponin I would be of great interest.

Structural Proteins

Filamin

Filamin was discovered in gizzard smooth muscle by Wang *et al*. (1975). It has an apparent M_r on SDS-PAGE of 250,000 and is abundant (30-40 % of the myosin concentration). Filamin is rod shaped, forms antiparallel end-to-end dimers and crosslinks actin filaments into bundles. (Reviewed in Small *et al*., 1986 and Marston and Smith, 1985). It is found in skeletal muscle (Betchtel, 1979) and in non-muscle cells (reviewed in Bretscher and Lynch, 1985).

Recently Dabrowska *et al*. (1985) demonstrated that filamin in molar ratios to actin of 1/30 to 1/200 activates actomyosin ATPase activity. It inhibits the ATPase at higher ratios. The presence of tropomyosin shifts the activation to higher filamin to actin ratios probably by competing with filamin for actin binding sites. The significance of the effect of filamin on actomyosin ATPase activity was questioned by Small *et al*. (1986). Using immunohistochemistry at both light and electron microscope levels, they discovered two domains in smooth muscle cells. One contained continuous longitudinal arrays of actin and myosin while the other contained filamin, actin and desmin in longitudinal fibrillar arrays, and α-actinin rich dense bodies. Due to the apparent absence of filamin from the actomyosin domain, Small *et al*. (1986) suggest that filamin is more

likely to be a regulator of tension maintenance through crosslinking of actin filaments than a regulator of actomyosin ATPase activity.

α-Actinin

Ebashi and Ebashi (1965) discovered a skeletal muscle protein that induced superprecipitation of actomyosin in the presence of ATP and named it α-actinin. This protein was found to cause gelation of actin filaments (Maruyama and Ebashi, 1965) and was localized to the Z-band of myofibrils (Masaki *et al.*, 1967). It is a rod shaped dimer of M_r 100,000 monomers and crosslinks actin filaments to form bundles (reviewed in Marston and Smith, 1985).

Immunofluorescence studies demonstrate that α-actinin is present in microfilament containing structures in non-muscle cells (reviewed in Bretscher and Lynch, 1985), and in the cytoplasmic dense bodies of smooth muscle cells (Small, 1985). The actin cross-linking properties of non-muscle α-actinin are inhibited by Ca^{2+} while those of the smooth muscle form are not (Burridge and Feramisco, 1981).

Vinculin and Talin

Vinculin was first purified from chicken gizzards (Geiger, 1979, and Feramisco and Burridge, 1980). It is a roughly spherical monomer with a Mr of 130,000 (Jockusch and Isenberg, 1981). Vinculin is found at

sites where actin filaments are attached to membranes, in non-muscle cells (Geiger, 1979) and in smooth muscle (Small, 1985). In smooth muscle it is present in continuous parallel ribs along the length of the cell which are associated with the plasma membrane plaques.

A protein that binds to vinculin, called talin, has been purified from chicken gizzards (Burridge and Connell, 1983). Talin also binds to a fibronectin receptor and may thus provide a linkage between actin flaments and the plasma membrane, although the precise mode of interaction between talin-vinculin and actin is not yet clear (Horwitz *et al.*, 1986).

Intermediate Filament Proteins

The intermediate filament proteins are broadly distributed in both muscle and non-muscle cells. This family of proteins consists of keratin found in cells of epithelial origin, vimentin and desmin with a broad distribution in most non-epithelial cells (including muscle), the neurofilament proteins, and glial filament protein. Sequence information indicates that these proteins all possess a conserved central sequence of α-helical coiled-coil, and variable head and tail regions involved in polymerization into filaments (Weber and Geisler, 1985). In smooth muscle the intermediate filaments can contain either desmin or vimentin or both (reviewed in Small and Sobieszek, 1983). They form part of a cytoskeletal network that also includes the

cytoplasmic dense bodies and the plasma membrane plaques (Somlyo and Franzini-Armstrong, 1985). Small *et al.* (1986) have indicated that filamin, actin, and α-actinin are also associated with this network. For a general review of intermediate filament proteins see Wang *et al.* (1985).

C. Summary and Aims

Tropomyosin, in conjunction with troponin, is an important regulatory protein in vertebrate striated muscle. Its role in smooth muscle is not well understood. The aim of this study was to search for and characterize tropomyosin binding proteins (hopefully troponin-like) in smooth muscle, so as to better understand the role of tropomyosin in this tissue. By 1980 two major pieces of evidence indicated that troponin might not be present in smooth muscle. (1) The actomyosin ATPase of vertebrate smooth muscle is regulated by myosin phosphorylation. (2) Troponin is not present in Ca^{2+} regulated actomyosin.

However, around the time that this study was started, several reputable scientists had suggested that myosin phosphorylation was either unimportant (Nonomura and Ebashi, 1980; Cole, Grand and Perry, 1982) or that additional control mechanisms were required (Driska *et al.*, 1981 and Persechini *et al.*, 1981). The ability of smooth muscle tropomyosin to greatly increase the activity of phosphorylated actomyosin indicated that it may be involved in a secondary control mechanism. Also, thin filaments had been prepared that could impart Ca^{2+} sensitivity to smooth muscle actomyosin (Marston *et al.*, 1980), providing evidence for a thin filament linked regulatory system.

At the outset of this work evidence for the absence of troponin from smooth muscle was weak. Several abundant proteins in the low M_r region of SDS-PAGE, where troponin

components were expected to be found, had not been purified or characterized. The absence of troponin from actomyosin preparations might be explained by its loss during purification. The possibility of a more labile troponin system was postulated for platelets by Côté and Smillie (1981). They noted that troponin I together with platelet tropomyosin acted as an efficient inhibitor of skeletal actomyosin ATPase. Calmodulin, in the presence of Ca^{2+} could release the inhibition by releasing troponin I-tropomyosin from the thin filament. One would not expect smooth muscle tropomyosin to dissociate from the thin filament. However, since smooth muscle tropomyosin activates rather than inhibits actomyosin ATPase, only the Ca^{2+} dependent dissociation of a troponin I-like inhibitor would be required for regulation. Another important piece of information was that smooth muscle tropomyosin had functionally retained its Ca^{2+} sensitive troponin binding site (Pearlstone and Smillie, 1982), and combined with troponin could effectively regulate skeletal actomyosin (Dabrowska *et al.*, 1980). We therefor thought that the pursuit of tropomyosin binding proteins in smooth muscle was a worthy cause.

While this study was in progress several important advances were made in the smooth muscle field. (1) Myosin phosphorylation was shown to be essential for activation of contraction, and more evidence accumulated for additional control mechanisms. (2) Caldesmon was purified and shown to

be capable of regulating contraction in a manner similar to that predicted from studies of troponin I and platelet tropomyosin. Its actual function though is not yet clear. (3) The amino acid sequence of gizzard tropomyosin was determined(Sanders and Smillie, 1985 and Helfman *et al.*, 1984). Its primary structure generally agreed with predictions made from troponin T binding studies (Pearlstone and Smillie, 1982). (4) Hirai and Hirabayashi (1983) used two dimensional gel electrophoresis to clearly separate all of the major components of gizzard smooth muscle.

In the work to be presented in this thesis, we have not found a troponin like protein. We have however purified and characterized a 21 kDa tropomyosin binding protein (SM21) and an abundant 22 kDa protein (SM22). Neither of these proteins has been previously characterized and both undoubtedly play an important role in smooth muscle function. In the process of purifying SM21 and SM22 we have also helped to sort out the identity of most of the relatively abundant low M_r proteins in smooth muscle and provided further evidence for the absence of low Mr troponin like components in these tissues.

II. General Materials and Methods

Procedures used in the characterization of both SM21 and SM22 are presented in this section. Those that are specific to one or the other of these proteins can be found in the appropriate chapter.

A. Gel Electrophoresis

SDS-PAGE was run using a Laemmli (1970) buffer system (Tris-glycine) with a 5 % stacking gel, and a range of separating gel concentrations. Several gel sizes were used during the course of this project. These included (1) a homemade apparatus for gels 1.5 mm thick and 6 cm in height, (2) three apparatuses from Bio-Rad, each having a gel thickness of 0.75 mm but with different heights (5.5, 10, and 13 cm). After electrophoresis, gels were stained with 0.25 % Coomassie Brilliant Blue R250 (Kodak) in 50 % methanol, 10 % acetic acid and destained in a 10 % methanol, 10 % acetic acid solution. Gels were photographed on a light box (fluorescent) through a yellow filter, using Panatomic-X film (ASA 32).

Gradient Gels

Separating gels with a 10-18 % gradient of polyacrylamide were poured with a device containing two conical chambers connected through a stopcock. The separating gel buffer in one chamber contained 18 % polyacrylamide and 15 % sucrose. An equal volume of buffer

containing 10 % polyacrylamide was present in the other chamber. The 18 % buffer was mixed with a Buchler vibrator and the system was drained from the chamber containing this buffer with a Pharmacia P1 peristaltic pump operating at 150 ml/h.

Two-dimensional Gel Electrophoresis

Non-equilibrium pH gradient electrophoresis (NEPHGE) was carried out by the method of O'Farrell *et al.* (1977). The NEPHGE solution consisting of 4 % acrylamide, 0.24 % N, N'-methylene-bis-acrylamide, 9 M urea, 2 % Nonidet P40, 2 % ampholines pH 3.5-10 (LKB), with 5 µl of N,N,N',N'-Tetramethylethylenediamine and 10 µl of ammonium persulfate (10 %) for each 5 ml, was suctioned into glass tubes of length 18 cm and internal diameter 1.5 mm. Tissue samples in 9 M urea were homogenized with a Sorval Omni-mixer, centrifuged in a Brinkman Eppendorf 5412 centrifuge for 5 min, loaded by Hamilton syringe, and electrophoresed for 3000 Vh from a positive upper tank electrode in 0.01 M H_3PO_4 to a negative lower tank electrode in 0.02 M NaOH. The NEPHGE gel was extruded into Laemmli gel running buffer and loaded onto 1.5 mm thick gels with a separating portion 13 cm in height and 14 % in polyacrylamide.

Gel Elution

Elution of proteins and peptides from SDS-polyacrylamide gels was carried out precisely as described by Hunkapiller *et al*. (1983), including the source of chemicals and the design of the elution apparatus. Proteins and peptides were separated on 10-18 % gradient gels with a thickness of 0.75 mm and a separating gel length of 10 cm. Stain time was 2 min. The dialysis membrane used was Spectropore 3 with an Mr cut-off of 3000.

Determination of Relative Levels of Actin, Tropomyosin, SM21, and SM22

One gram of tissue, either bovine artery or chicken gizzard, was homogenized in 5 ml of 8 M urea, 2 % SDS, 0.2 % mercaptoethanol and 0.6 M Tris-HCl, pH 6.8. The homogenate was centrifuged and the supernatant loaded in a dilution series on SDS-polyacrylamide gels. These included 10-18 % and 13 % gels, 13 cm in height for SM22, and 13 % gels, 5.5 cm in height for SM21. After electrophoresis the gels were stained as above. Gel scanning was carried out with a Joyce Loebl Chromoscan 3 scanning densitometer. The relative dye binding capacities of SM21, SM22, tropomyosin and actin were determined by scanning a dilution series of these purified proteins. Each protein standard was made up in a stock solution of known concentration (amino acid analysis) prior to dilution with Laemmli gel loading buffer. The dye binding capacities of the purified proteins relative to tropomyosin

(1.00) were SM22 (1.16), actin (0.69) and SM21 (0.62). These values were taken from the portion of the absorbance vs. concentration curve that obeyed the Beer-Lambert law, as were all the readings from muscle extracts.

B. Circular Dichroism

The circular dichroic (CD) spectrum of a protein arises from differential absorption of left and right (A_L and A_R) circularly polarized light. This differential absorption also imparts ellipticity to plane polarized light. It has become convention to express CD as ellipticity through the equation:

$$\Theta = 2.303(A_L - A_R)180/4\pi \text{ degrees} \qquad (1)$$

CD arising from the peptide backbone is measured primarily in the far-UV region of 190-230 nm. It is useful for comparative purposes to convert the resulting Θ values to mean residue ellipticities ($[\Theta]$) through the equation:

$$[\Theta] = \Theta M_o/10.1c \qquad (2)$$

where M_o = mean residue weight (calculated from amino acid composition), 1 = the cell path length in cm, and c = the protein concentration in g/ml. The CD spectrum of proteins in the far-UV depends primarily on their secondary structure. In a simple form the ellipticity at any one wavelength can be expressed as the sum of the contribution from α-helix, β-sheet and random coil.

$$[\Theta(\lambda)] = X_\alpha[\Theta_\alpha(\lambda)] + X_\beta[\Theta_\beta(\lambda)] + X_r[\Theta_r(\lambda)] \qquad (3)$$

where X_α, X_β, and X_r represent the predicted fraction of each structure and $[\theta_\alpha(\lambda)]$, $[\theta_\beta(\lambda)]$ and $[\theta_r(\lambda)]$ are values that represent the known intensity of each structural type at a given wavelength. The relative intensities are based on the CD spectra of five proteins that have had their structures previously determined by X-ray crystallography (Chen *et al.*, 1974). For SM21 and SM22 we solved these equations using 3 wavelengths, 215, 220, and 225 nm. Provencher and Glockner (1981) have developed a basis spectrum from 16 proteins of known structure, and included β-turn as a fourth structural entity. We have used this basis spectrum in conjunction with the Contin version I computer program to further analyse the structural content of SM21 and SM22. The input to the program was the mean residue ellipticities at 1 nm intervals from 190-230 nm.

The near-UV CD spectrum of most proteins derives mainly from the aromatic residues, tryptophan, tyrosine and phenylalanine. They possess a low level of CD while free in solution which can be greatly enhanced in the presence of nearby asymmetric structure or by interaction with other aromatic residues (Strickland 1974). It is the convention of some authors to convert near-UV [θ] values back into CD values through the equation

$$\Delta\epsilon = \epsilon_L - \epsilon_R = [\theta]N/3300 \qquad (4)$$

where ϵ_L and ϵ_R are the molar extinction coefficients of

left and right circularly polarized light and N is the number of amino acids in the protein. Equation (4) can be derived from equations (1), (2) and the Beer-Lambert law (Cantor and Schimmel, 1980). One of the reasons for this conversion is that aromatic residues only represent a fraction of the total residues in proteins.

The near-UV CD absorption bands are assigned according to their relation to specific π-π^* electronic transitions of benzene. (eg, ^1L_B). Both the ground and excited state can exist in a variety of vibrational states which are assigned numbers according to energy level (eg; 0-0 indicates that both the π and π^* states have 0 vibrational energy (Strickland 1974).

CD spectra of SM21 and SM22 were determined on a Jasco J500c spectropolarimeter. The machine was calibrated with aqueous solutions of (1) (d)-10-camphor sulphonic acid and (2) pantoyl lactone.

C. Hydrodynamics

We have used hydrodynamics to determine the molecular weight, shape and subunit composition of SM21 and SM22.

Ultracentrifugal analyses

Ultracentrifugal studies were performed at 20°C in a Beckman Spinco Model E analytical ultracentrifuge equipped with an RITC temperature-control unit and electronic speed control. Optical alignment and focussing were carried out according to manufacturer's instructions. Photographic plates were analysed on a Nikon Model 6C microcomparator.

Sedimentation Velocity

Sedimentation coefficients were measured at 60,000 rpm from schlieren photographs taken through a quartz window at constant time intervals. Kel F 2° centerpieces (12 mm) were used. Protein concentrations were determined by amino acid analysis. The sedimentation coefficients were calculated by the maximum ordinate method from the schlieren photographs and corrected to the standard conditions of water at 20°C ($s_{20,w}$) according to Svedberg and Pederson (1940). The $s°_{20,w}$ was determined by extrapolating values of $s_{20,w}$ to infinite protein dilution.

Sedimentation Equilibrium

Weight-average molecular weights were measured using conventional low-speed sedimentation equilibrium

techniques (Richards *et al.*, 1968). Double-sector charcoal-filled Epon centerpieces (12 mm) were employed. Measurements were made using either Rayleigh interference optics, or for concentrations < 1 mg/ml, UV absorbance. Protein samples were dialysed at least 48 h at 4°C to ensure complete equilibration. Initial protein concentrations of these samples were determined in duplicate using synthetic boundary runs (Richards *et al.*, 1968). Equilibrium experiments were then performed with 0.1 ml of protein solution, using rotor speeds chosen from graphical data (Chervenka, 1969).

Equilibrium photographs were measured manually at a magnification of 50x. Several vertical readings of fringe displacement (y) were taken at equally spaced horizontal points (x) that were no more than 100 μm apart. About 20 data points were collected. The horizontal x-readings were converted to values of radial distance (r) using the position of the counterbalance reference hole (r = 7.28 cm) and the camera magnification factor. The apparent weight average molecular weight (M_w) was calculated from the protein concentration (c) as a function of r according to:

$$M_w = [2RT/(1-\bar{v}\rho)\omega^2].[d\ln c(r)/dr^2] \qquad (5)$$

where R is the universal gas constant, T is the experimental temperature (K), $\bar{v}$ is the partial specific

volume of the protein determined from amino acid composition according to Cohn and Edsall (1943), ρ is the solvent density and ω is the angular velocity.

The Stokes radii of SM21 and SM22 were calculated from the equation:

$$R_{s,sed} = M_w(1-\bar{v}\rho)/6N\pi\eta_o s^o_{20,w} \qquad (6)$$

where η_o is the solvent viscosity in poise.

Analytical Gel Chromatography

Analytical gel filtration experiments were carried out on a 7.5 mm x 600 mm TSK G3000 SW high performance liquid chromatographic column. The column was run at 0.5 ml/min with a Spectra-Physics SP 8750 pump and the effluent monitored at 230 and 280 nm with a Micrometrics 788 dual wavelength variable detector. Samples (1-2 mg/ml) were loaded in 20 μl. The void volume (Vo) was determined with ferritin and the total volume (V_T) with mercaptoethanol. The partition coefficient (σ) was calculated from the elution volume (Ve) by the relationship

$$\sigma = (Ve-Vo)/(V_T-Vo) \qquad (7)$$

The Stokes radius from gel filtration (R_s,gel) of SM22α was then calculated from a standard curve of log R_s vs. σ (Siegel and Monty, 1966). The following proteins with known

Stokes radii were used as standards: β galactosidase, 69 Å ; catalase, 52 Å ; lactate dehydrogenase, 41 Å ; hemoglobin, 24 Å ; myoglobin, 19.8 Å ; cytochrome c, 17,2 Å (compiled from Byers and Kay (1982) and Seigel and Monty (1966).

Axial Ratio

In order to get some concept of the shape of SM21 and SM22, we determined the frictional ratio (f/f_{min}), or the ratio of the observed frictional coefficient to that of an idealized spherical particle of identical M_w:

$$f/f_{min} = R_s/R_o = R_s/[3\ M_w\ \bar{v}/4\ \pi\ N]^{1/3} \qquad (8)$$

where R_s is the Stokes radius determined from either sedimentation or gel filtration or an average of the two, and R_o is the Stokes radius of the equivalent unhydrated sphere. Proteins are normally hydrated. If the degree of hydration is known, or can be estimated, the contribution due to asymmetry alone can be evaluated (Oncley, 1941) by:

$$f/f_o = (f/f_{min})/[1 + (w/\bar{v}\rho)]^{1/3} \qquad (9)$$

where w is the hydration expressed in grams of water per gram of protein. Amino acid composition was used for the estimation of w (Kuntz and Kauzmann, 1974). When f/f_o is known the axial ratio of a protein can be estimated from the tables of Schachman (1959). Since we have not carried out the appropriate viscosity measurements we can not distinguish between a prolate or an oblate ellipse. The axial ratios in this thesis are given arbitrarily as those of a prolate ellipse.

D. Amino Acid Analyses

For amino acid analysis, a fired test tube containing the protein sample in constant boiling HCl with 0.1 % phenol was evacuated, sealed and incubated at 110°C for 24 h. The hydrolysate was dried over NaOH pellets in a vacuum desiccator and analysed on either a Durram model D-500 or Dionex model D-502 amino acid analyser. Half-cystine was determined as cysteic acid after oxidation in performic acid as described by Moore (1963), and as carboxymethyl cysteine following treatment with iodoacetic acid (Crestfield *et al.*, 1963). The carboxymethylation was scaled down in order to treat samples smaller than 5 mg. The cysteic acid color factor was determined from a standard (Pierce) while the color factor for aspartic acid was used for carboxymethyl cysteine. Tryptophan was determined after hydrolysis in methane sulphonic acid (Simpson *et al.*,(1976). The pH of hydrolysates prepared by this method was adjusted to ≈ 2.2 with 5 M NaOH prior to analysis. The color factor of tryptophan was determined from a standard (Pierce). Proteins of known tryptophan content (actin and troponin T) were also analysed in order to ensure that the techniques were working properly.

E. Proteins

Rabbit skeletal troponin, troponin I, troponin T, T2 fragment of troponin T (residues 159 to 259), troponin C, myosin and actin were kindly provided by the L.B. Smillie laboratory. Calmodulin was purified according to Walsh (1978), and was also supplied by K. Golosinska, according to Gopalakrishna and Anderson (1982). Chicken gizzard tropomyosin was purified according to Smillie (1982). Nucleic acids were removed from tropomyosin preparations by chromatography on DEAE-cellulose (Whatman-52) at pH 7.6 (Woods, 1969).

III. The Tropomyosin Binding Proteins of Smooth Muscle:Purification of SM21 Under Denaturing Conditions

A. Introduction

Tropomyosin is a two stranded coiled coil. It belongs to the group of α-fibrous proteins which includes keratin, myosin, epidermin and fibrinogen (reviewed in Smillie, 1979). Proteins in this group are noted for their high degree of asymmetry. This asymmetry results from stabilization of a linear α-helical structure by interchain hydrophobic and ionic interactions.

Two types of tropomyosin polypeptide chain are present in mammalian cross striated muscle. These are designated α and β based on their mobility on SDS-PAGE (Cummins and Perry, 1973). Amino acid sequencing has indicated that each muscle tropomyosin polypeptide chain consists of 284 amino acids (Mak *et al.*, 1980). The sequence consists of a series of equivalent but not identical 7 residue repeats, that provide the structural basis for the interchain coiled coil structure (McLachlan and Stewart, 1975). Analyses of the tropomyosin sequence (McLachlan and Stewart, 1975), supported by physical and chemical data, indicate that its α-helical chains are parallel and in register, and that overlap between tropomyosin molecules occurs in a head to tail manner with an ∝ 9 residue overlap. A further quasi-equivalent repeat consisting of 14 regions, each 19.66 amino acids in length can be interpreted as correlating with

actin binding sites (7 strong homostrand and 7 weak heterostrand) (McLachlan and Stewart, 1976).

Tropomyosin binds to actin in a cooperative manner (Wegner, 1979). This binding along with head to tail polymerization (measured by viscosity) can be greatly reduced by removal of 11 residues from the COOH-terminus (Mak and Smillie 1981), indicating the importance of the head to tail overlap region. Besides self assembly and binding to actin, tropomyosin also binds troponin in a 1:1 ratio. In the absence of Ca^{2+}, troponin holds tropomyosin in a position on the thin filament that has a negative effect on actomyosin ATPase activity. Addition of Ca^{2+} alters the troponin tropomyosin interaction so as to move tropomyosin into a position more favorable to actomyosin ATPase activity (Phillips *et al.*, 1986). This shift in the position of tropomyosin is the first movement detectable by time resolved X-ray diffraction, during skeletal muscle activation (Kress *et al.*, 1986).

Troponin binds to tropomyosin at two sites (see Fig. 2). One site involves the elongated NH_2-terminus of troponin T binding to the head to tail overlap region of tropomyosin (reviewed by Mak and Smillie, 1981). For a different point of view see Ohtsuki *et al.* (1986). This site is relatively insensitive to Ca^{2+} and is believed to anchor troponin to the thin filament. The second site involves the binding of troponin I and the COOH-terminus of troponin T to tropomyosin at $\simeq$ 1/3 the distance from its COOH-terminus in

the region of cysteine 190 (reviewed in Pearlstone and Smillie, 1983). Binding at this site is highly Ca^{2+} sensitive and is believed to be involved in the regulation of the actomyosin ATPase. To obtain Ca^{2+} regulation under physiological conditions all of the troponin subunits and tropomyosin must be present (reviewed in Leavis and Gergely, 1984).

In smooth muscle, actin, myosin and tropomyosin are present in abundance. Troponin, however has not been found. Smooth muscle actomyosin ATPase activity is controlled primarily by MLCK. What then is the role of tropomyosin in smooth muscle ?

Analysis of smooth muscle by x-ray diffraction indicates that tropomyosin is present on the thin filament (Vibert 1972). Sequence studies of both chicken gizzard smooth muscle tropomyosins (α and β)[1] reveal some fundamental similarities with their rabbit skeletal muscle counterparts (Helfman *et al.*, 1984 ; Sanders and Smillie, 1985). The chain length of both smooth muscle forms is 284 amino acids. The heptapeptide repeat is largely conserved indicating similar intersubunit interactions and the quasi-equivalent actin binding sites are also present (Sanders and Smillie, 1985).

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[1] I have named gizzard smooth muscle tropomyosin according to the skeletal muscle forms that are alternatively spliced from the same gene (ie. α gene for α skeletal and α smooth). Due to the anomalous behavior of tropomyosin on SDS-PAGE several names exist in the literature. The α form has been called γ (Sanders and Smillie, 1985) and β (Helfman *et al.*, 1984).

Three significant alterations occur in the sequence of smooth muscle tropomyosin relative to its skeletal muscle counterpart (Fig. 3). The COOH-terminal residues from 258-284 are greatly altered in both α and β chains. This is the region that binds the NH_2-terminal portion of troponin T in skeletal muscle and contributes to head-to-tail polymerization. Sanders and Smillie (1984) have shown that smooth muscle tropomyosin has greater viscosity and thus greater head-to-tail interaction than its skeletal counterpart. It is also known that under physiological conditions, skeletal muscle tropomyosin can only potentiate actomyosin ATPase activity in the presence of troponin and Ca^{2+} whereas the smooth muscle form does not require troponin. The viscosity of skeletal tropomyosin is increased in the presence of the NH_2-terminal portion of troponin T (Jackson *et al.*, (1975). Since smooth muscle tropomyosin does not bind this portion of troponin T (Pearlstone and Smillie, 1982), it is tempting to consider that its altered COOH-terminus is a compensating factor which allows it to potentiate actomyosin ATPase activity in the absence of troponin. However, platelet tropomyosin, which polymerizes poorly and inhibits actomyosin ATPase activity (Côté and Smillie, 1982) has the smooth muscle type COOH-terminus (Lewis *et al.*, 1983). There must therefor be some other functional purpose for this region.

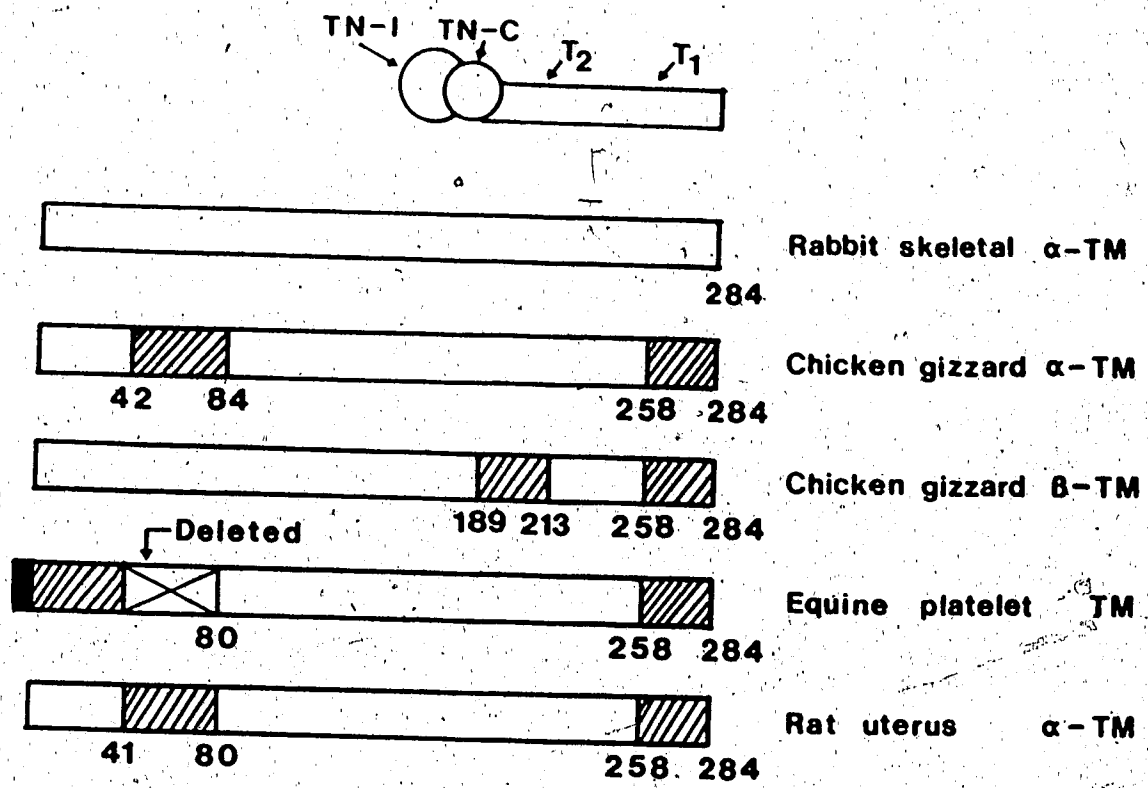


FIG. 3. Schematic comparison of various tropomyosin amino acid sequences. Clear areas represent regions of strong homology between all sequences. Hatched areas represent regions with distinctly different sequences compared to rabbit skeletal α-tropomyosin. In the case of platelet tropomyosin, 42 amino acids are deleted from the NH_2-terminal side of residue 80 but the precise location of the deletion is uncertain. Five additional residues are also present at the NH_2-terminus . A schematic of troponin is also shown at the top of the figure, over the portion of tropomyosin with which it is thought to interact. The amino acid sequences can be found in Sanders and Smillie 1985, with the exception of rat uterus α-tropomyosin which is from Ruiz Opazo *et al.*, 1985. Abbreviations are TN-I, troponin I ; TN-C, troponin-C ; T1 and T2, amino acids 1 to 158 and 159 to 259 of troponin T respectively. [Adapted from a figure kindly provided by L.B. Smillie].

A second alteration in amino acid sequence occurs in the β isoform of smooth muscle tropomyosin between residues 189 and 213. The introduction of 3 acidic residues at 201, 211 and 213 corrects an anomaly present in the quasi-equivalent actin binding repeat which had been attributed to the imposition of structural requirements for troponin T binding. However, Pearlstone and Smillie (1982) have demonstrated that gizzard tropomyosin can bind the COOH-terminal portion of troponin T, and troponin can function with this tropomyosin (Dabrowska *et al.*, 1980). The third major alteration occurs in the sequence of amino acids 48 to 80 in the α chain. Some of the changes in this region lead to a theoretical destabilization of interchain bonds which could lead to local unfolding. Presently, no proteins are known that bind to this region of tropomyosin.

Recent sequencing of cDNAs and genomic DNAs coding for tropomyosin has led to the realization that the differences between smooth and skeletal tropomyosins result largely from alternative splicing of specific exons (Helfman *et al.*, 1986 ; Macleod *et al.*, 1985 ; Ruiz-Opazo *et al.*, 1985). This splicing is highly tissue specific which indicates that important functional characteristics are being selected. At present we do not know enough about smooth muscle contractile proteins to state what these functions are. It is therefor important to further investigate the role of tropomyosin in smooth muscle. We have approached this problem by searching for tropomyosin binding proteins.

We used 3 methods for identifying tropomyosin binding proteins. These were (1) copurification, (2) affinity chromatography and (3) binding of ^{125}I-tropomyosin to proteins that had been electrophoretically separated on denaturing gels (often referred to as a gel overlay). With the first method we isolated a 21 kDa protein from bovine aorta. The second method gave negative results, while the third method indicated the presence of several tropomyosin binding proteins and formed the basis of a large part of the present work.

The gel overlay procedure used in this study was developed by Carlin *et al.* (1980) in order to identify calmodulin binding proteins in brain. The general method has also been used to identify proteins that bind to actin (Snabes *et al.*, 1981) and vinculin (Otto, 1983). Using the gel overlay as an assay we have purified a 21kDa tropomyosin binding protein from chicken gizzard smooth muscle under denaturing (8M urea) conditions.

B. Materials and Methods

Radioiodination of Gizzard Tropomyosin

Chicken gizzard tropomyosin was labeled with ^{125}I using lactoperoxidase. The technique was modified from Eaton *et al.* (1975) in order to increase the relative amount of ^{125}I incorporated per mg of tropomyosin. The labeling solution (1.5 ml) contained 1.5 mg of chicken gizzard tropomyosin, 30 μg of lactoperoxidase (Sigma), 3 mCi of ^{125}I, 0.15 M KCl and 0.05 M sodium phosphate (pH 7.0). The reaction was initiated by addition of 7 μl of 0.06% H_2O_2. This addition was repeated 3 times at 10 minute intervals and the reaction was terminated by addition of 30 μl of 50 mM DTT and 10 μl of 0.05 M KI. The resulting ^{125}I-tropomyosin was dialyzed against 3 one litre changes of 0.15 M KCl, 0.05 M sodium phosphate, 1 mM dithiothreitol (pH 7.0), for 8 hr each. The level of incorporation of ^{125}I into tropomyosin ranged from 0.5 to 1.0 mCi/mg.

Binding of ^{125}I-tropomyosin to Proteins on Denaturing Gels

SDS-polyacrylamide gel electrophoresis was run according to Laemmli (1970) using 0.75 mm thick gels 10 cm in height. Whole tissue samples were prepared by extracting 1 g of tissue with 10 ml of 1.5% SDS, 8 M urea, 50 mM $NaPO_4$, pH=7.0, 1.5% mercaptoethanol. After electrophoresis, the gels were fixed in 25% isopropanol, 10% acetic acid for 4 h with 4 changes. They were then washed in buffer A (0.15 M

KCl, 0.05 M imidazole, 1 mM DTT (pH 7.0) for 6 h with 4 changes. The gels were further washed for 2 h with buffer A containing 1 mg/ml of bovine serum albumin (fraction V, Sigma). They were then incubated in 5 to 15 ml of buffer A containing 25-50 μg of ^{125}I tropomyosin. Next, the gels were washed in buffer A for 24 h, 6 changes. They were then fixed in 10% acetic acid, 10% methanol for 30 min, stained with 0.25% Coomassie Brilliant Blue R-250 (Kodak) in 50% methanol, 10% acetic acid for 10 min, destained, and dried onto Bio-Rad filter paper backing. The gels were exposed for 12-24 h on Kodak XAR-5 X-ray film at -20°C using a Dupont Cronex enhancing screen. ^{125}I-calmodulin binding experiments were conducted with a protocol similar to ~~that used~~ for ^{125}I-tropomyosin. Scanning of gels and autoradiographs was carried out on a Joyce-Loebl Chromoscan 3 scanning densitometer.

Purification of SM21; Denaturing Method

All purification procedures were carried out at 4°C unless otherwise stated. One kg of chicken gizzard muscle was homogenized in 7 l of buffer containing 1 M KCl, 50 mM imidazole, pH 7.0, 0.25 mM phenylmethylsulfonyl fluoride, 0.2 μg/ml leupeptin, 0.2 μg/ml pepstatin, 0.1 mg/ml soy bean trypsin inhibitor, 1 mM dithiothreitol, 1 mM EGTA. Homogenization was carried out in 4 batches, in a 3 l Waring blender for 3 x 15 sec at high speed. The homogenate was centrifuged at 16,000 g for 60 min. The supernatant (~5 l)

was then dialyzed for 2 x 10 h against 2 x 25 l of double distilled H_2O containing 0.02% mercaptoethanol. The dialysate was then centrifuged at 16,000 *g* for 30 min. The precipitate was resuspended in 2 l of buffer containing 0.2 M KCl, 0.05 M imidazole, pH 7.0, 1 mM DTT and stirred for 1 h. The suspension was centrifuged at 16,000 *g* for 30 min. The resulting supernatant was fractionated between 25 and 60% saturated $(NH_4)_2SO_4$. The 60% $(NH_4)_2SO_4$ precipitate was dialyzed against distilled H_2O containing 0.02% mercaptoethanol and lyophilized. Two g of this lyophilized powder were then dissolved in 200 ml of buffer containing 8 M urea, 50 mM Tris, pH 8.0, 1 mM DTT at 22°C. The resulting solution was centrifuged at 27,000 *g* for 20 min and the supernatant was loaded on a 5 x 30 cm column of DEAE cellulose (Whatman DE22) previously equilibrated in the same buffer. The column flow rate was 70 ml/h. The flow through fraction from this column was dialyzed against distilled H_2O and lyophilized. This material was then fractionated on a column of CM-cellulose (Whatman CM32) as described in Fig. 9.

C. Results

Purification of SM21 from Bovine Aorta

Both tropomyosin and tropohin can be obtained from a high salt extract of a skeletal muscle acetone powder. We made an acetone powder from 500 g of minced bovine aorta according to Smillie (1982). The powder (90 g) was extracted overnight (4°C) with 1 l of 0.6 M LiCl, 50 mM Tris-HCl, pH 7.5, 1 mM DTT. The pH of the extract was lowered to 4.6 with HCl and the resulting precipitate was collected by centrifugation and freeze dried. It contained tropomyosin, actin, bands with mobility similar to troponin I and troponin T, and several bands of higher Mr (Fig. 4 A, lane 1). The pH 4.6 precipitate was dissolved in a solution containing 8 M urea, 1 mM DTT, 50 mM Tris pH 7.5, and loaded on a column of DEAE-Sephadex (Pharmacia-A25) which had previously been equilibrated with the same buffer. The bands comigrating with troponin T and I were present in the DEAE flow through (Fig. 4 A, lanes k and m). A band with mobility slightly greater than troponin T could now also be distinguished. The DEAE flow through was chromotagraphed on CM-cellulose in 8M urea pH 4.7 (Fig. 4 B). Three main peaks were obtained, each containing one of the 3 proteins that comigrate with troponin T or I (Fig. 4 A, lanes b, c, d-f, h-j). The latter protein (aorta SM21), pooled from fractions 115-125, was further characterized by amino acid analysis (Table I). Its amino acid composition indicated

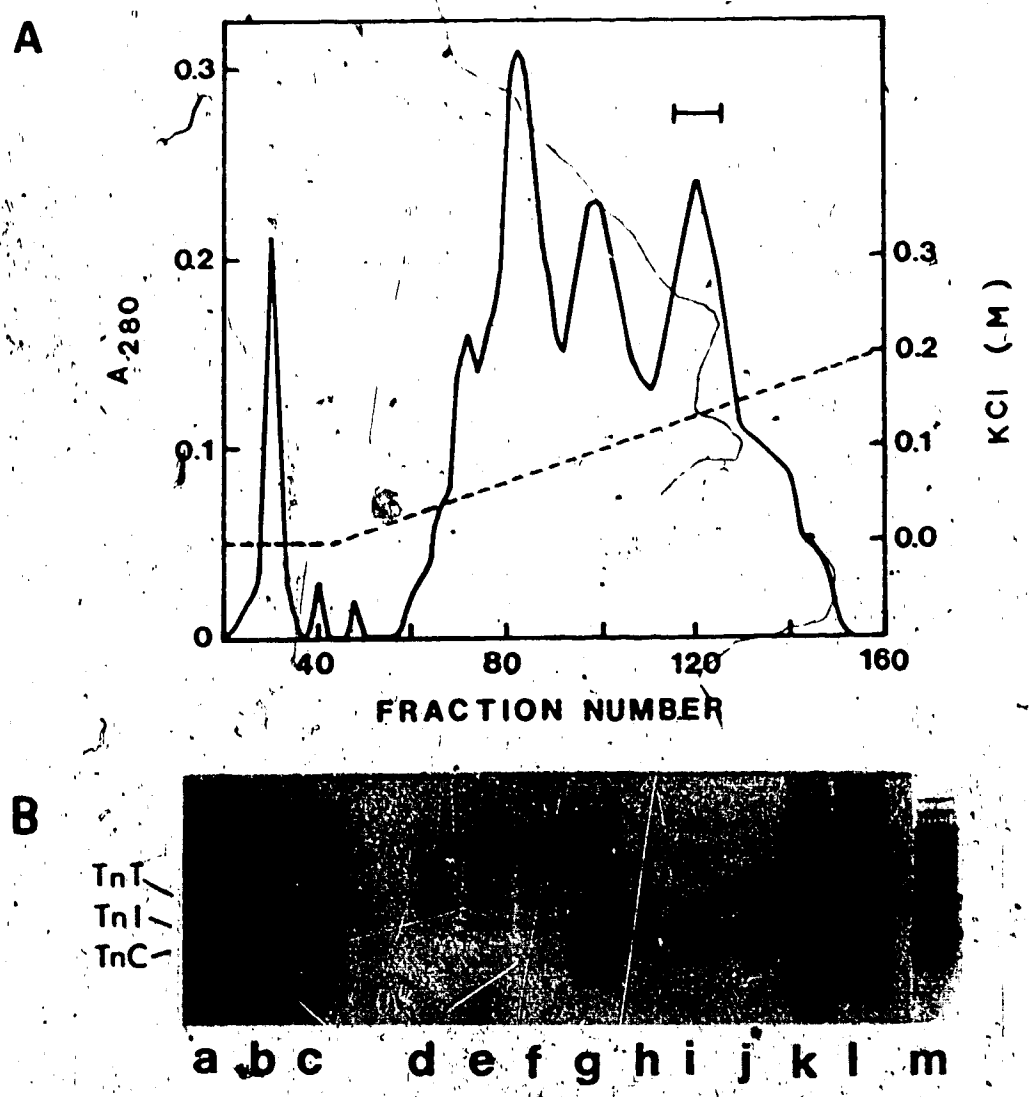


FIG. 4 Purification of Aorta SM21. (A) A SDS-polyacrylamide gel using the buffer system of Weber and Osborn (1969) and including 6 M urea in both gel and buffer. The gel was 8 % in acrylamide, 6 cm in height and 1.5 mm thick. Lanes are (a and g) rabbit skeletal troponin ; (b and c) fraction 82 : 10 and 30 µl ; (d to f) fraction 96 : 10, 30 and 20 µl ; (h to j) fraction 120 : 10, 30 and 20 µl ; (k and m) DEAE flow through ; (l) pH 4.6 precipitate. (B) The chromatographic profile represents the loading of 0.08 g of lyophilized DEAE flow through in 25 ml of buffer (8 M urea, 1 mM DTT, 50 mM sodium acetate, pH 4.7) on a column (2.5 x 22 cm) of CM-cellulose (CM-32) previously equilibrated with the same buffer. The column was then developed with 400 ml of the same buffer, followed by a 1 l linear NaCl gradient (0 to 0.25 M). Flow rate, 35 ml/h ; fraction size, 7 ml.

TABLE I

Amino acid composition of SM21 (mole/mole)

The composition of SM21 is based on an integral number of alanines in order to approach an apparent M_r of 21,000. Samples of SM21 were hydrolyzed for 24, 48 and 72 h. Values for serine and threonine were extrapolated to 0 time. Valine and isoleucine were taken from the average of 48 and 72 h hydrolysates.

Residue	Chicken gizzard SM21D[a]	Chicken gizzard SM21N[b]	Bovine aorta SM21	Rabbit skeletal muscle TNI[c]	Rabbit skeletal muscle TnT[d]
Asx	11	11	12	15	20
Thr	7.2	7.6	8.9	3	6
Ser	14	14	14	10	9
Glx	18	18	21	33	57
Pro	6.8	7.4	8.1	5	n
Gly	33	33	27	8	
Ala	16	16	15	14	26
Val	9.6	9.5	11	7	11
Met	3.1	3.4	1.7	9	5
Ile	5.1	5.1	5.0	5	8
Leu	8.2	7.9	9.7	17	19
Tyr	9.6	10.4	8.0	2	4
Phe	6.5	6.9	7.2	3	5
His	5.1	5.4	7.3	4	6
Lys	23	24	21	24	39
Arg	5.0	5.2	7.2	15	25
Trp	1.8[e,f]	2.0[f]	n.d.[g]	1	2
Half-Cys	14[e,f]	14[f]	n.d.[g]	3	0
Total	199	201	202[h]	178	259
M_r	21,000	21,200	21,400	20,700	30,522

[a] SM21 purified under denaturing conditions.

[b] SM21 purified under non-denaturing conditions.

[c] Composition of troponin I from its sequence (Wilkinson and Grand, 1978).

[d] Composition of troponin T from its sequence (Pearlstone et al., 1977).

[e] Determined after performic acid oxidation as cysteic acid (Moore, 1963).

[f] Determined after carboxymethylation (Crestfield et al., 1963) as carboxymethyl cysteine.

[g] n.d. - not determined.

[h] The total number of residues given for aorta SM21 assumes no changes in Half-Cys or Trp content compared to gizzard SM21.

that it was basic, in agreement with its interaction with ion exchange resins. Other notable features were a high glycine content and a qualitatively high half-cystine content. After further purification by gel elution, the NH_2-terminal amino acid sequence of aorta SM21 was determined by automated sequence analysis (Fig. 5). When SM21 was dialysed from a solution of 8 M urea to a solution containing 0.1 M KCl, 10 mM Tris, pH 7.0, 1 mM DTT it precipitated, leaving only trace amounts of protein in solution. Efforts to keep this protein in solution by adding tropomyosin or calmodulin were unsuccessful.

^{125}I-Tropomyosin Gel Overlay

In order to locate tropomyosin binding proteins in smooth and skeletal muscle, we electrophoresed muscle extracts on SDS-polyacrylamide gels and then probed the gels with ^{125}I-tropomyosin from gizzard smooth muscle. In chicken breast skeletal muscle we were able to identify three tropomyosin binding proteins that comigrate on gels with myosin heavy chain, troponin-T and troponin-I (Fig 6 A to 6 C, lane a). Binding to myosin heavy chain occurred only when it was present in large amounts as is illustrated by the barely detectable reaction in the skeletal muscle standard when compared to whole skeletal muscle (Fig. 6 A to 6 C ; lanes a and b). ^{125}I-tropomyosin was also shown to bind to purified rabbit skeletal troponin-T and -I (Fig. 6 A to 6 C ; lane b).

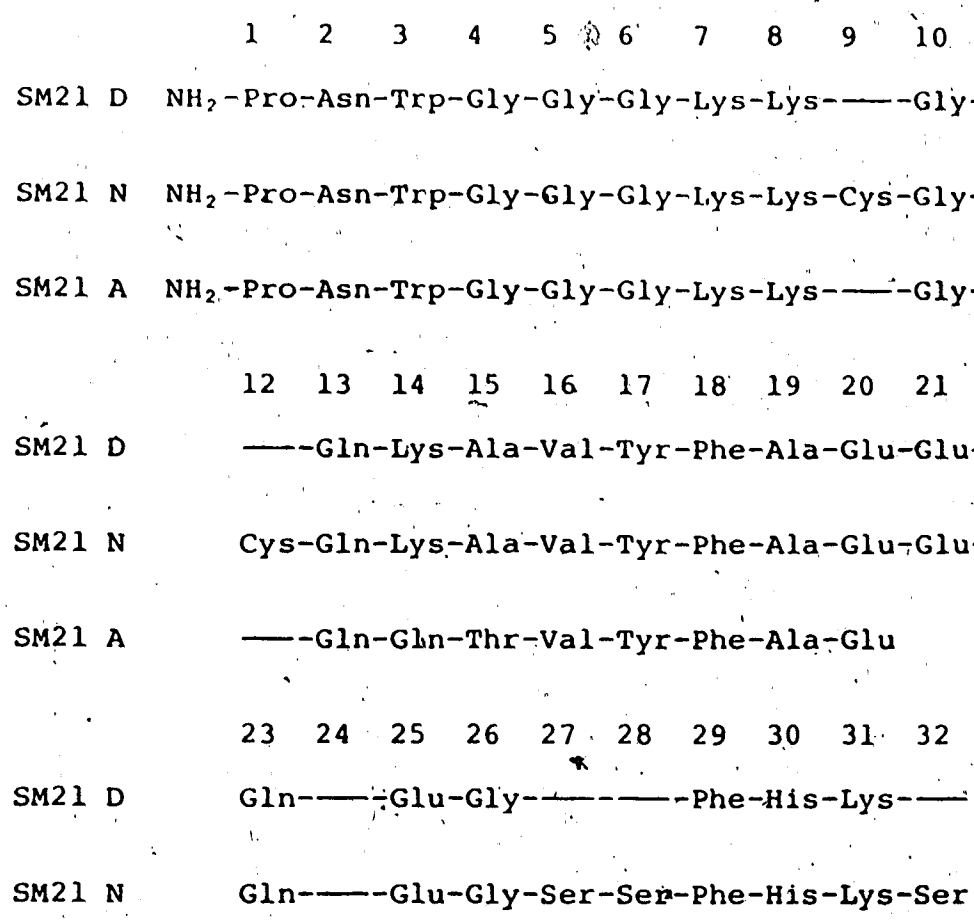


FIG. 5. NH_2-terminal amino acid sequence of SM21. After purification under denaturing conditions (SM21 D) SM21 (50 nmol) was sequenced on a Beckman model 890 B Sequencer extensively modified as described (Wittmann-Liebold *et al.*, 1976 ; Wittmann-Liebold, 1980 and Hunkapiller and Hood, 1978). The repetitive yield was 92.6 %. For SM21 D, phenylthiohydantoin derivatives of Ser or Cys or their degradation products were observed at cycles 9, 12, 24, 27, 28 and 32 but no decision could be made between them. Two further samples were sequenced for confirmation. SM21 N (750 pmol of protein purified under non-denaturing conditions) was carboxymethylated (Crestfield *et al.*, 1963) and sequenced on an Applied Biosystems 470 A Gas Phase Sequencer according to Hewick *et al.* (1982). The repetitive yield was 88.8 %. Two further samples were analysed for confirmation. Residue 24 could not be clearly identified. SM21 A (10 nmol of aorta SM21) was sequenced in the same manner as SM21 D. The repetitive yield was 90.0 %.

FIG. 6. Binding of ^{125}I-tropomyosin to muscle extracts and standards on SDS-polyacrylamide gels. The method of binding is given in Materials and Methods with approximately 75 μg of protein being loaded in lanes a, c, d, e and g and 10 μg in lanes b and f. The separating gel had dimensions of 10 cm (height), 12 cm (width) and 0.75 mm (depth), and contained a 10 to 18% polyacrylamide gradient. (A) A Coomassie blue stained gel of muscle extracts and standards. (B) A gel similar to A that has gone through the ^{125}I-tropomyosin binding procedure and has been dried down onto filter paper. (C) An autoradiograph of B. Lanes include (a) SDS-urea extract of chicken breast skeletal muscle, (b) rabbit skeletal muscle standards including myosin (M), actin (A), troponin-T (TnT), troponin-I (TnI) and troponin-C (TnC), (c) SDS-urea extract of chicken gizzard smooth muscle, (d) gizzard smooth muscle not extracted by 1 M KCl, (e) proteins extracted from gizzard smooth muscle by 1 M KCl and precipitated by dialysis against distilled H_2O, (f) calf thymus histones, from top to bottom H1 (doublet), H3, H2A, H2B, H4, (g) proteins extracted from gizzard by 1 M KCl and soluble after dialysis against distilled H_2O. The apparent M_r of the protein standards are myosin 200,000, actin 42,000, TnT 39,000, H1 30,000, TnI, 22,000, TnC, 20,000, H3 18,000 and H4 14,000. They are indicated by(▸). Additional proteins designated in lane g ; gel (A), include tropomyosin (Tm), SM2 (S) and myoglobin (Mb). (●▸) indicates the position of SM21.


A
B
C
M
TnT
H1
TnI
TnC
H3
H4
a
b
c
d
e
f
g

The primary tropomyosin binding protein in chicken gizzard smooth muscle migrates to a position corresponding to a M_r of 21,000 (Fig. 6 A to 6 C ; lane c). Several other proteins in smooth muscle, including myosin, give a weak response in the whole extract. Further investigations were aimed at purification and characterization of the 21 kDa protein (SM21).

Purification of SM21 from Chicken Gizzard

The first step in purifying SM21 was to extract gizzards with 1 M KCl. This extract was then dialyzed against distilled H_2O and the resulting precipitate was pelleted and run, along with the supernatant, on denaturing gels (Fig. 6 A to 6 C ; lanes e and g). The results indicate that, with the exception of some myosin and a 28 kDa protein, most of the tropomyosin binding activity was extracted with 1 M KCl (Fig. 6 A to 6 C ; lane d). When this extract was dialyzed against distilled H_2O , most of the tropomyosin binding proteins precipitated (Fig. 6 A to 6 C ; lane e). Three of the tropomyosin binding proteins in the distilled H_2O precipitate comigrate with calf thymus histones H1, H3 and H4 which were also shown to bind tropomyosin (Fig. 6 A to 6 C ; lanes e and f ; and Fig. 7 A to 7 C). H2A and H2B are lost from the polyacrylamide gels during the washing procedure. Other proteins that are lost from the gel during the ^{125}I-tropomyosin binding procedure include myoglobin and tropomyosin (Fig. 6 A to 6 C ; lanes c and g).

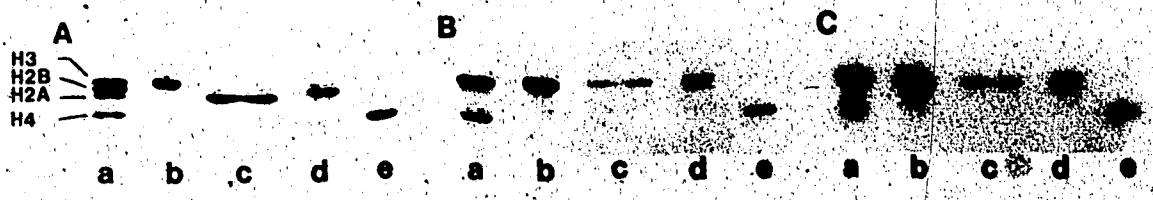


FIG. 7. Binding of ^{125}I-tropomyosin to core histones on SDS-polyacrylamide gels. Gel specifications are as in Fig. 6 A - C. lanes are (a) core histone, (b) H3, (c) H2A, (d) H2B, (e) H4. Each histone is loaded at 2 μg.

SM21 was separated from the histones by extraction with 0.2 M KCl. This procedure leaves chromatin undissolved along with actomyosin (Fig. 8, lane c) while solubilizing SM21 and proteins tentatively identified from their M_r as SM22, tropomyosin, α-actinin, MLCK, caldesmon, filamin and actin (Fig. 8, lanes e and f). SM21 was found to precipitate between 25 and 60% saturated $(NH_4)_2SO_4$. It then flowed through a DEAE-cellulose column at pH 8.0 in the presence of 8 M urea. The DEAE flow through was then fractionated on CM-cellulose at pH 4.7 in the presence of 8 M urea (Fig. 9). The peaks containing SM21 and a 22 kDa protein (SM22) were separately pooled and run on denaturing gels (Fig. 10). The 60% $(NH_4)_2SO_4$ precipitate in Fig. 10 A to 10 C ; lane a, illustrates a case where SM21 and SM22 have not been clearly separated by electrophoresis. This is a fairly common problem with SM21 on longer gels and can be remedied by running mini gels as in Fig. 12. Despite the lack of electrophoretic separation in Fig. 10 A to 10 C; lane a, it can be clearly seen in lanes b and c that it is SM21 and not SM22 that is binding ^{125}I-tropomyosin. The yield of SM21 from this preparation is approximately 40 mg/kg. Its maximum solubility in 0.2 M salt solution, pH 7.0 was found to be approximately 0.2 mg/ml. The amino acid composition of SM21 is shown in Table I. The most outstanding features include high levels of glycine and half-cystine. The composition of SM21 is not similar to that of either troponin I or troponin T.

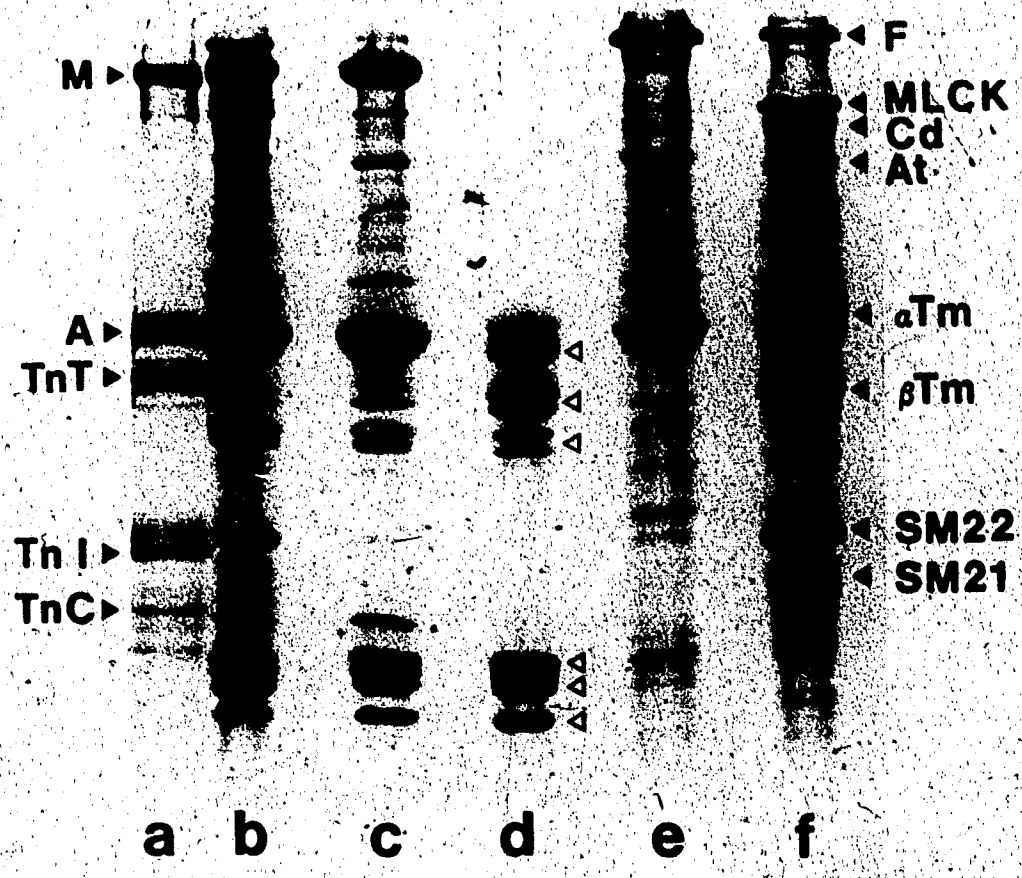


FIG. 8. SDS-polyacrylamide gel electrophoresis of fractions at various stages of SM21 purification. Gel specifications are as in Fig. 6. Lanes are (a) rabbit skeletal muscle standards as in Fig. 6; (b) distilled H_2O precipitate; (c) material not extracted by 0.2 M KCl; (d) from top to bottom, α and β tropomyosin from gizzard, and histones H1, H3, H2A and H2B, and H4 from calf thymus; (e) 25% $(NH_4)_2SO_4$ precipitate; (f) 60% $(NH_4)_2SO_4$ precipitate. Identification of F, filamin ; Cd, caldesmon ; MLCK ; and At, α-actinin in lane f was made tentatively from relative mobilities, according to Feramisco and Burridge (1980) and Ngai *et al*. (1984).

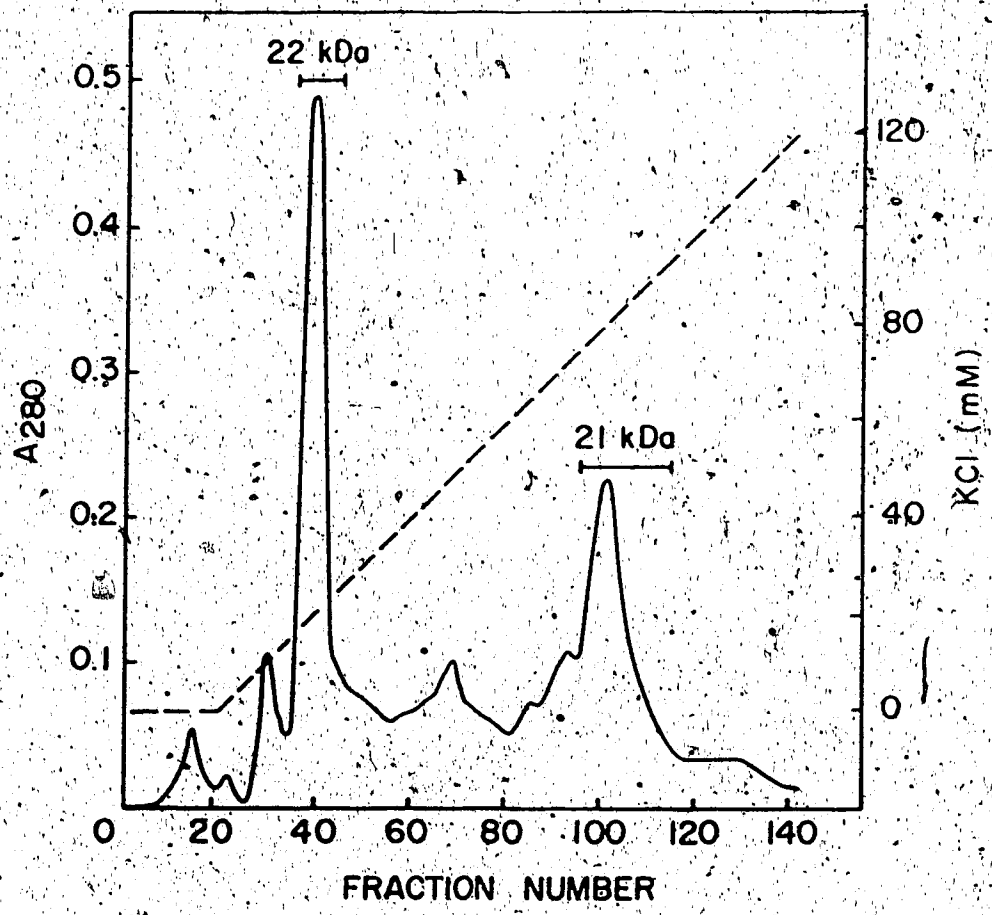


FIG. 9. CM-cellulose chromatographic purification of SM21. The chromatographic profile represents the loading of 0.3 g of lyophilized DEAE flow through in 30 ml of buffer (8 M urea, 50 mM sodium acetate, pH 4.7, 1 mM DTT, 22°C) on a column (2.5 x 22 cm) previously equilibrated with the same buffer. The column was then developed with 200 ml of the same buffer followed by a 1.5 l linear NaCl gradient (0 to 0.15 M). Flow rate, 35 ml/h; fraction size, 10 ml.

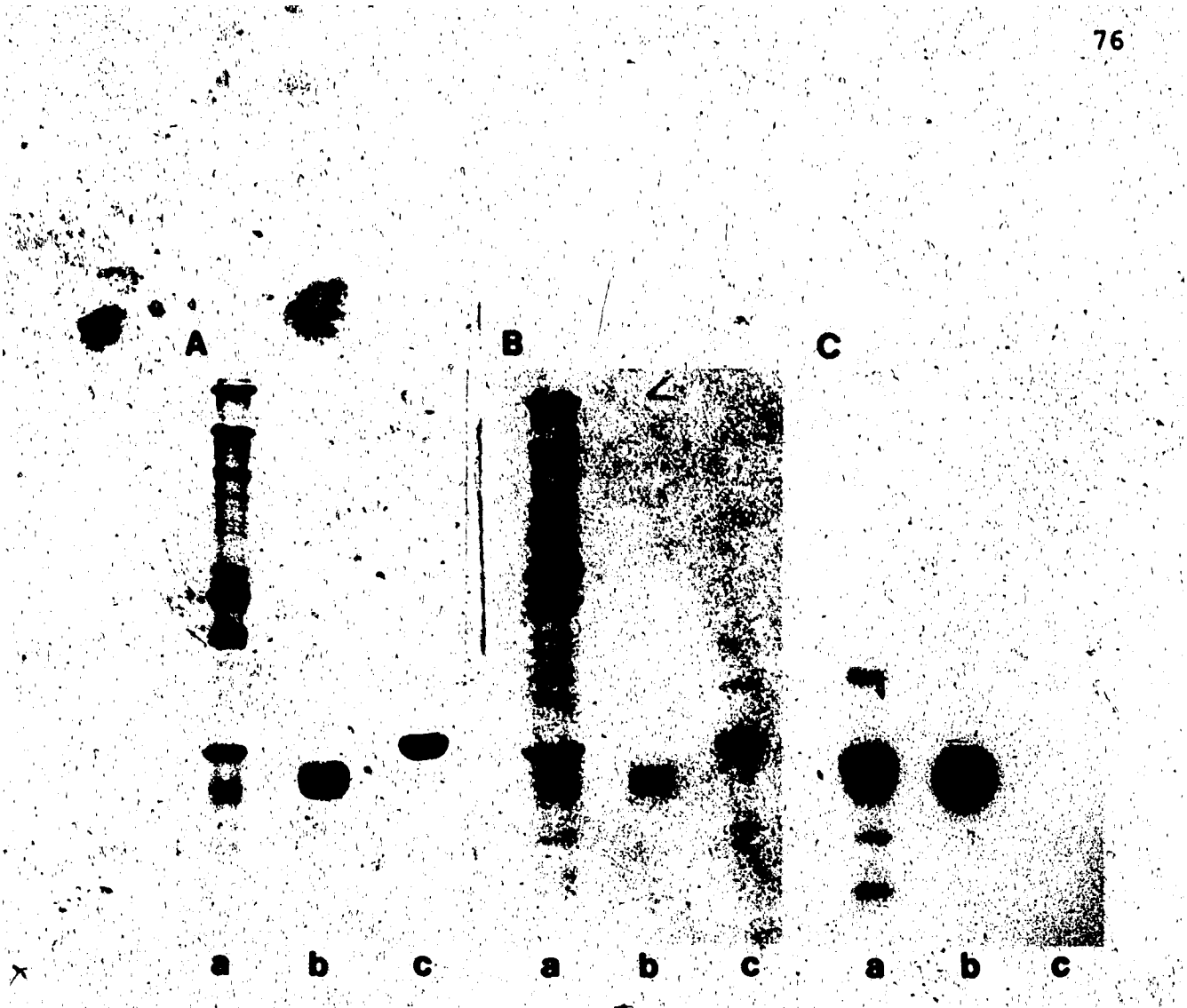


FIG. 10. Binding of ^{125}I-tropomyosin to SM21 purified under denaturing conditions. Gel specifications are as in Fig. 6 A to 6 C. Lanes are (a) 50 μg of the 60% $(NH_4)_2SO_4$ precipitate, (b) 4 μg of the SM21 containing peak in Fig. 9; (c) 4 μg of the SM22 containing peak from Fig. 9. (A) coomassie blue stained gel, (B) coomassie blue stained gel that has been subjected to the overlay procedure, (C) autoradiograph of B.

Given an apparent M_r for SM21 of 21,000, amino acid analysis indicates that it contains 33 basic residues (Lys, Arg, His) and a total Asx, Glx content of 29 residues. This finding suggests that SM21 is strongly basic. Evidence to back this finding comes from 2-dimensional gel electrophoresis (Fig. 11) which demonstrates that SM21 is considerably more basic than SM22 or myoglobin which have pIs in the range of 8.0 to 8.5 (Hirai and Hirabayashi, 1983 and chapter 5). From its mobility on 2-dimensional gels we estimate the pI of SM21 to be in the range of 9.5 to 10. We have determined the sequence of the 30 NH_2-terminal amino acid residues of SM21 which had been purified under denaturing conditions but not carboxymethylated (Fig. 5). We have also partially sequenced a cyanogen bromide fragment from SM21 that was purified by gel elution. Ten nmol were sequenced on a Beckman 890 B Sequencer. The amino acid sequence obtained was

Ala-Gln-Lys-Val-Gly-Gly-Gly-Asp-Gly-Gly-Pro-Ala-Val-Gly-Gly.

The repetitive yield was 78.5 %.

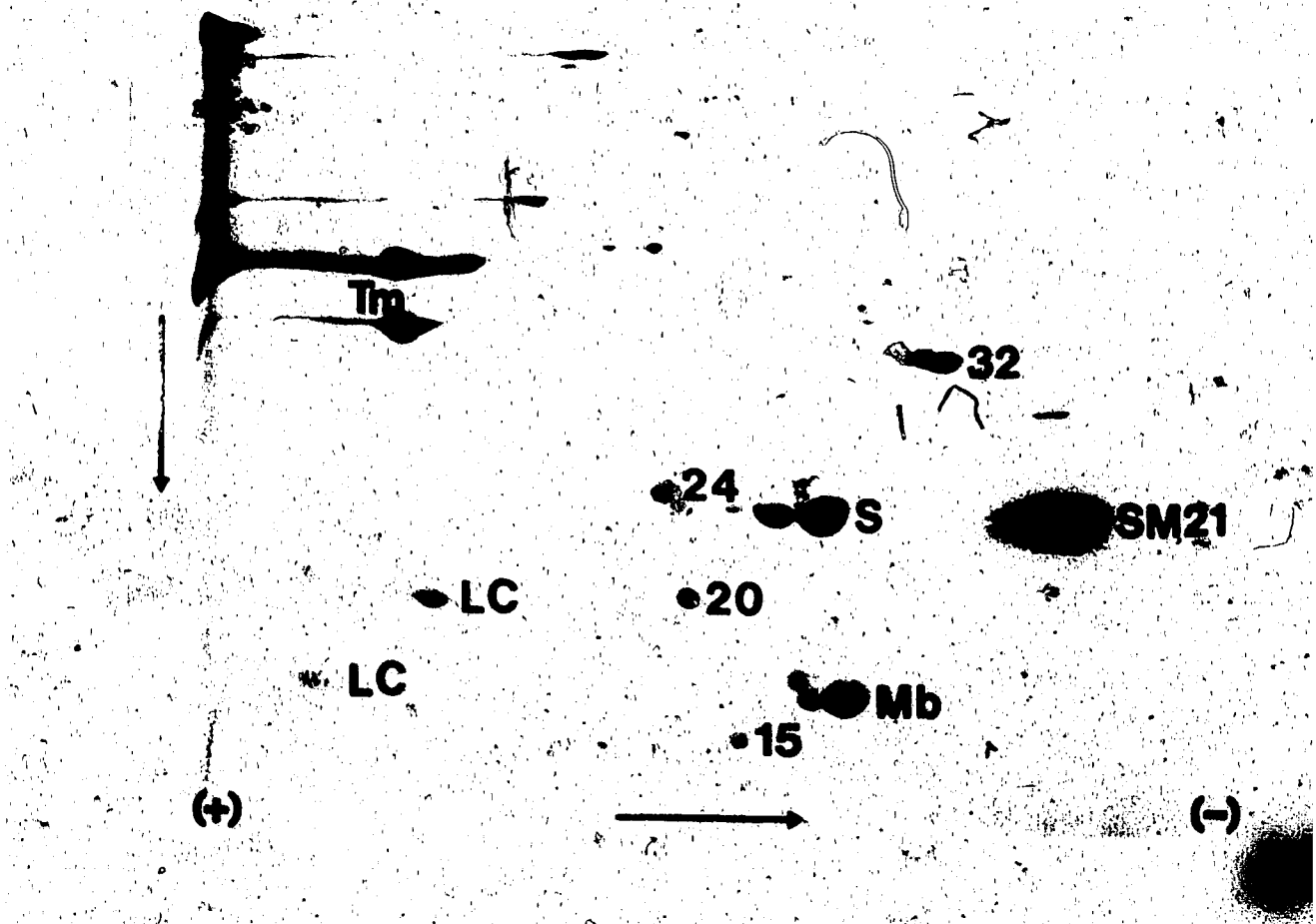


FIG. 11. Two-dimensional gel of a 9 M urea extract of gizzard smooth muscle plus 5 μg of purified SM21. The horizontal dimension is non-equilibrium isoelectric focusing carried out for 2500 Vh in the presence of pH 3-10 ampholines (LKB). The vertical dimension is SDS-polyacrylamide gel electrophoresis. Tm, tropomyosin ; S, SM22 ; Mb, myoglobin ; LC, 20 and 17 kDa myosin light chains. Identification of spots was made according to Hirai and Hirabayashi (1983). The numbers 32, 24, 20, 15 indicate the presence of 4 medium abundance basic proteins in smooth muscle of unknown function or composition.

D. Discussion

Our first attempt at finding a tropomyosin binding protein in smooth muscle involved searching for proteins that copurify with tropomyosin from an acetone powder of bovine aorta. With a skeletal muscle acetone powder, isoelectric precipitation of tropomyosin from a high salt extract that also contains troponin does not result in co-precipitation of troponin. In aorta smooth muscle, however, this procedure does precipitate three basic proteins, two with gel mobilities similar to troponin T and one similar to troponin I. While we have not characterized the two forms that co-migrate with troponin T, we can guess that the lower mobility protein is glyceraldehyde 3-phosphate dehydrogenase. Sanders *et al.* (1986) have demonstrated that this enzyme is present in smooth muscle and shares common antigenic determinants with troponin T. It has a mobility on SDS-PAGE similar to that of troponin T and is basic. The higher mobility protein may correspond to SM32 (See Fig. 11 and chapter 4).

The protein from the pH 4.6 precipitate with a mobility similar to troponin I was further characterized and shown not to resemble either troponin I or T in amino acid composition, NH_2-terminal amino acid sequence, or in solubility properties in the presence of tropomyosin and calmodulin. We temporarily discontinued the project but soon encountered SM21 again in chicken gizzard smooth muscle.

Our next endeavour was the ^{125}I-tropomyosin overlay. When we electrophoresed a whole chicken skeletal muscle extract on a denaturing gel and probed the gel with ^{125}I-tropomyosin (chicken gizzard), we found that it bound to troponin T, troponin I and myosin. Some explanations for the lack of binding to actin include the possibility that actin was not renatured or that it did not polymerize, making binding by a low concentration of tropomyosin (0.15 μM) unlikely. Binding to myosin occurred only when it was present in gross excess and is unlikely to be significant. Most important, a reaction occurred with troponin T and troponin I. We therefore believe that our system should be capable of locating similar components in smooth muscle if they exist.

The main tropomyosin binding protein in a SDS-urea extract of gizzard smooth muscle is SM21. This protein is present in a molar ratio to actin of ≃1.0:16 (see chapter 4) which is relatively low compared to the 1.0:7.0 ratio of troponin to actin in skeletal muscle. SM21 however, gives a stronger binding reaction than either troponin T or troponin I (Fig. 6 A to 6 C ; lanes a and c)

When gizzard smooth muscle was extracted with 1 M KCl and the extract dialyzed against distilled H_2O, a precipitate formed that contained cytoskeletal elements and chromatin. ^{125}I-tropomyosin bound to both SM21 and histones in this precipitate. The binding of platelet tropomyosin to a histone has been demonstrated previously (Stewart *et al.*,

1983). The significance of this reaction is not clear. When the distilled H_2O precipitate was washed with a 0.2 M salt solution, SM21 was extracted along with several other proteins that have gel mobilities similar to proteins known to be involved with the microfilament network including filamin, caldesmon, myosin light chain kinase, α-actinin, actin and tropomyosin (Fig. 8 ; lane f). This finding provides some evidence for the interaction of SM21 with the contractile apparatus. It is also possible that SM21 interacts with chromatin but is extracted at a lower salt concentration than the histones.

We were able to purify SM21 from the 0.2 M salt extract by $(NH_4)_2SO_4$ fractionation and ion exchange chromatography in the presence of 8 M urea. The last major contaminant to be separated from SM21 was the abundant basic protein SM22. We were able to clearly demonstrate that it is the minor SM21 component of whole gizzard extracts and not the abundant SM22 band that is binding ^{125}I-tropomyosin by chromatographing the individual CM-cellulose peaks containing these proteins on denaturing gels followed by an ^{125}I-tropomyosin binding assay (Fig. 10).

The late elution of SM21 from CM-cellulose indicates that it is a basic protein. Further evidence for this was provided by non-equilibrium 2-dimensional gel electrophoresis. Amino acid analysis of SM21 also indicated that it is a basic protein. Other aspects of SM21's amino acid composition indicated that it was not similar to either

troponin T or troponin I. The greatest differences were in the glycine and half-cystine content as well as in the total charge. The N-terminal amino acid sequence of SM21 (30 residues) bears no resemblance to any part of troponin-T or troponin-I and is not significantly similar to any amino acid sequence in the National Biomedical Research Foundations protein sequence data base. In addition, antibodies made against SM21 did not cross-react with troponin components in an ELISA assay nor did antibodies to troponin-T or troponin-I cross-react with SM21 (unpublished results). SM21 purified in the presence of 8 M urea was soluble to a maximum of 0.2 mg/ml in physiological salt buffer.

This brings us back to the protein with mobility similar to troponin I that we first isolated from bovine aorta. It is clear from amino acid composition and NH_2-terminal amino acid sequence that this protein is homologous to SM21 from gizzard. Indeed, when the procedure for purifying SM21 from gizzards was applied to bovine aortas, up to precipitation with 70 % saturated $(NH_4)_2SO_4$, a considerable enrichment of a ~~21 kDa~~ tropomyosin binding protein was detected (Fig. 12). We made attempts, using both western blotting and gel overlay, to determine the tissue distribution of SM21 (unpublished data), but only managed to clearly demonstrate its presence in chicken gizzard. In retrospect, the use of partial purification from each tissue, rather than SDS-urea extracts may be a better method

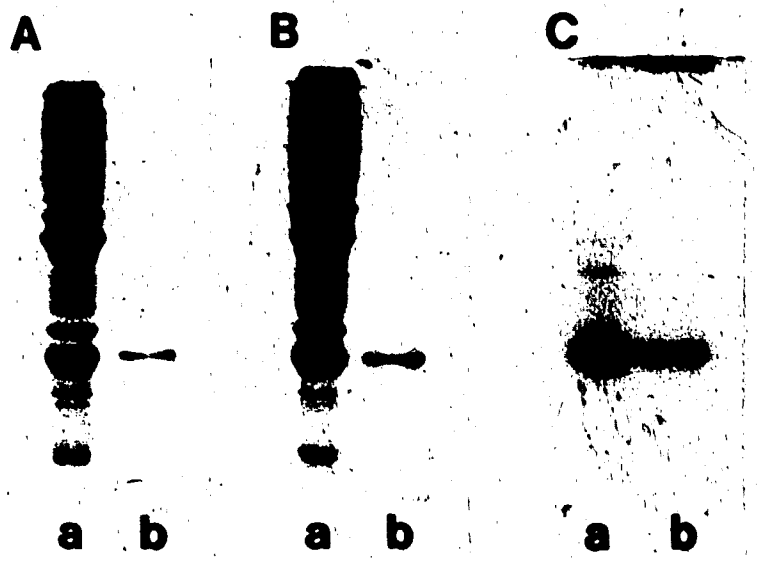


FIG. 12. Binding of ^{125}I-tropomyosin to bovine aorta SM21. The separating gel contained 13% polyacrylamide and had dimensions of height (5.5 cm), width (3 cm) and depth (0.75 mm). (A) coomassie blue stained gel, (B) A gel similar to A which was stained with coomassie blue following the ^{125}I-tropomyosin binding procedure, (C) an autoradiograph of B. Lane (a) 25 μg of a 60% $(NH_4)_2SO_4$ precipitate from bovine aorta. (b) 0.5 μg of chicken gizzard SM21.

for investigating SM21's tissue distribution. Immunofluorescence microscopy may also be a useful technique for this purpose.

We also carried out a series of binding studies. We found that SM21 would bind to tropomyosin and troponin I affinity columns and could not be removed by 0.6 M salt, but was removed with 0.6 M salt and 8 M urea (unpublished result). SM21 also bound calmodulin by gel overlay (Chap. 4 ; Fig. 18). We used ELISA to demonstrate that SM21 could bind an alkaline phosphatase labeled goat immunoglobulin directed against rabbit immunoglobulin. The binding to immunoglobulin was inhibited by non-immune serum or tropomyosin but not by troponin I, troponin T or calmodulin (unpublished result). In light of our identifying SM21 on denaturing gels and then purifying it by denaturing methods, we wondered if these binding results had any significance. Our approach to this problem was a non denaturing purification of SM21.

IV. Purification of SM21 under Non-denaturing Conditions and Physical Characterization

A. Introduction

In order to purify SM21 under non-denaturing conditions we have chosen to start with a myofibril preparation. A smooth muscle version of this preparation was introduced by Sobieszek and Bremel (1975). It can be used for the purification of filamin, myosin, caldesmon, MLCK, intermediate filament proteins, actin, tropomyosin, and actomyosin (reviewed in Small and Sobieszek, 1980). Ngai *et al.* (1984) modified the myofibril preparation for purification of caldesmon and MLCK under near physiological solvent conditions. They extracted these proteins from myofibrils with a buffer containing 25 mM Mg^{2+} and then purified them by column chromatography. Since SM21 was already known to co-purify with myofibrillar proteins we adapted the myofibril-high Mg^{2+} extract procedure for purification of SM21. We were then able to physically characterize it using hydrodynamics and circular dichroism.

The second aim of this chapter was to attempt to gain a better understanding of the binding properties of SM21 by modifying the conditions of the ^{125}I-tropomyosin overlay and by carrying out ^{125}I-calmodulin and ^{125}I-SM21 overlays.

B. Materials and Methods

Non-denaturing Purification of SM21

Fresh gizzard smooth muscle (450 gm) was homogenized in 1.8 l of wash buffer (20 mM Tris, pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 0.05% Triton X-100) in a 3 l Waring blender for 3 x 15 sec. The homogenate was centrifuged at 16,000 *g* for 30 min. The pellet was then resuspended in 2 l of wash buffer without Triton X-100 and centrifuged as above. This latter step was repeated once. The pellet was then suspended in 2.0 l of 40 mM Tris (pH 7.5), 100 mM NaCl, 25 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA and 0.25 mM phenylmethylsulfonyl fluoride, homogenized for 15 sec and centrifuged at 16,000 *g* for 30 min. The supernatant was then concentrated to 200 ml with a Minitan tangential flow filtration device (Millipore) using a PTGC filter. This step typically required 6 h. The Militan retentate was then dialyzed against 8 l of buffer containing 45 mM potassium phosphate, pH 6.45, 1 mM EGTA and 1 mM DTT, for 8 h and loaded on a column of CM-cellulose (CM 32) as described in Fig. 15. Column fractions containing SM21 were pooled and fractionated on Affi-gel Blue (BIO-RAD) (Fig. 16). The salt concentration of the column eluant was monitored with a Radiometer conductivity meter.

Characterization of SM21

Native SM21 was carboxymethylated prior to sequencing. Its NH_2-terminal amino acid sequence was determined on an

Applied Biosystems 470A Sequencer according to Hewick *et al.* (1981).

For ultracentrifugation studies SM21 was dialysed against a buffer containing 0.1 M KCl, 50 mM sodium phosphate, pH 7.0 and 1 mM DTT. Analytical gel filtration was carried out in KMED (0.1 M KCl, 0.05 M 3-(N-morpholino)propanesulfonic acid, 1 mM EGTA, 1 mM DTT, pH 7.0) buffer. Circular dichroism was carried out in 50 mM phosphate buffer, pH 7.0 with 1 mM DTT. In some cases 0.1 M KCl was added for CD analysis between 210 and 230 nm or 0.1 M KF was added for CD analysis between 190 and 230 nm.

Gel Overlay

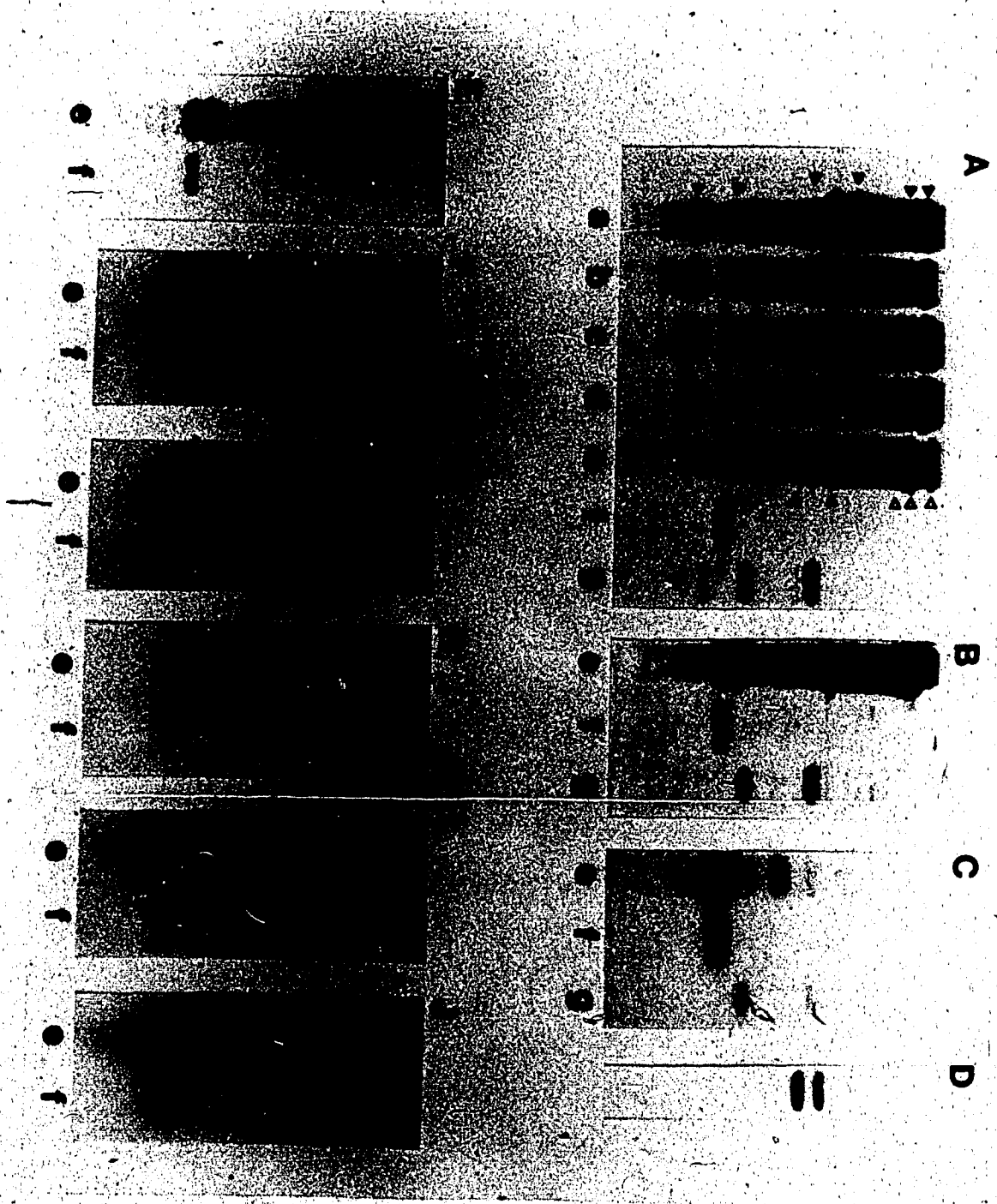
Several of the gel overlays in this chapter were carried out on mini gels (5.5 cm in height), in order to obtain higher resolution of SM21. Labeling of SM21 and bovine brain calmodulin with ^{125}I was carried out in the same manner as described for tropomyosin in chapter 3. Calmodulin was labeled to $\simeq$ 1 mCi/mg and SM21 to $\simeq$ 0.25 mCi/mg. The overlay experiments with these proteins were also identical to those previously described for tropomyosin, using 2 μg/ml of ^{125}I-calmodulin or 10 μg/ml of SM21. ^{125}I-calmodulin binding experiments were carried out in the presence of either 1 mM EGTA or 1 mM Ca^{2+}.

C. Results

Non-Denaturing Purification of SM21

We began this purification by producing myofibrils in a manner similar to that described by Ngai *et al.* (1984), a major difference being the lack of 40 mM NaCl in the wash buffer. The material extracted by the wash steps included relatively large amounts of myosin, actin, tropomyosin, SM22 and myoglobin (Fig. 13 A; lane b). SM21 was extracted in good yield from the myofibrils by a high Mg^{2+} buffer (Fig. 13 A; lane e and Fig. 14, and Table II). The molar ratio of actin to SM21 in this extract is 0.7:1.0 as compared to 16:1.0 in a SDS-urea extract of whole gizzard smooth muscle (Fig. 14). Other proteins greatly enriched by this procedure include filamin, caldesmon, and myosin light chain kinase. SM22 is enriched to a lesser extent due to larger losses in the preceding washes, while tropomyosin is present in minimal amounts (Fig. 14). Material largely not extracted by the Mg^{2+} extract includes myosin, α actinin, desmin, actin, tropomyosin, a 32 kDa band which has not, to our knowledge, been previously mentioned in the literature and histones (Fig. 13 A; lane d). The high Mg^{2+} extract was concentrated using a Militan filtration apparatus and then chromatographed on CM-cellulose at pH 6.5 (Fig. 15). While this method results in separation of SM21 from most major contaminants, further purification on Affi-gel blue was required to obtain SM21 that was >95% pure (Fig. 16).

FIG. 13. Non-denaturing purification of SM21. (A) A SDS gel, stained with coomassie blue, containing 13% polyacrylamide with length 5.5 cm and thickness 0.75 mm. Lanes include (a) SDS-urea extract of gizzard smooth muscle,(▸) indicates from top to bottom, myosin, myosin light chain kinase, desmin, tropomyosin, SM21 and myoglobin, (b) first low salt Triton X-100 wash of gizzard smooth muscle,(◂) indicates SM24 and SM20, (c) material not extracted after 3 low salt washes (myofibrils), (d) material not removed from myofibrils after high Mg^{2+} extraction,(▷) indicates 20 kDa MLCand 17 kDa MLC, (e) high Mg^{2+} extract,(◁)indicates from top to bottom, filamin, caldesmon, α-actinin, actin, 32 kDa band, and SM22, (f) 2 μgm of SM21 purified under non-denaturing conditions, (g) 5 μgm of rabbit skeletal troponin. The band designations are made according to (Sobiezek and Bremel, 1975 ; Small and Sobieszek, 1980 ; Hirai and Hirabayashi, 1983 ; Ngai *et al.*, 1984 ; Walsh *et al.*, 1983 and Murakami and Uchida, 1985). (B) A gel that has been subject to the ^{125}I-tropomyosin binding procedure as described in Materials and Methods and stained with coomassie blue; (C) an autoradiograph of B; (D) Autoradiograph of chicken gizzard tropomyosin that has been ^{125}I-labeled with lactoperoxidase and electrophoresed on a polyacrylamide gel; (E) Similar to B; (F to J) Autoradiographs of gels similar to E that have been incubated in buffer containing 0.15 μM ^{125}I-tropomyosin, 0.15 M KCl, 50 mM imidazole, pH 7.0, 1 mM DTT; (F) No addition; (G) Plus 3 μM unlabeled tropomyosin; (H) Plus 20 μM SM21; (I) Plus 0.5 M KCl; (J) Autoradiograph of a gel similar to E that has been incubated with 0.15 μM ^{125}I-tropomyosin in the presence of the high Mg^{2+} extraction solution (see Methods).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D

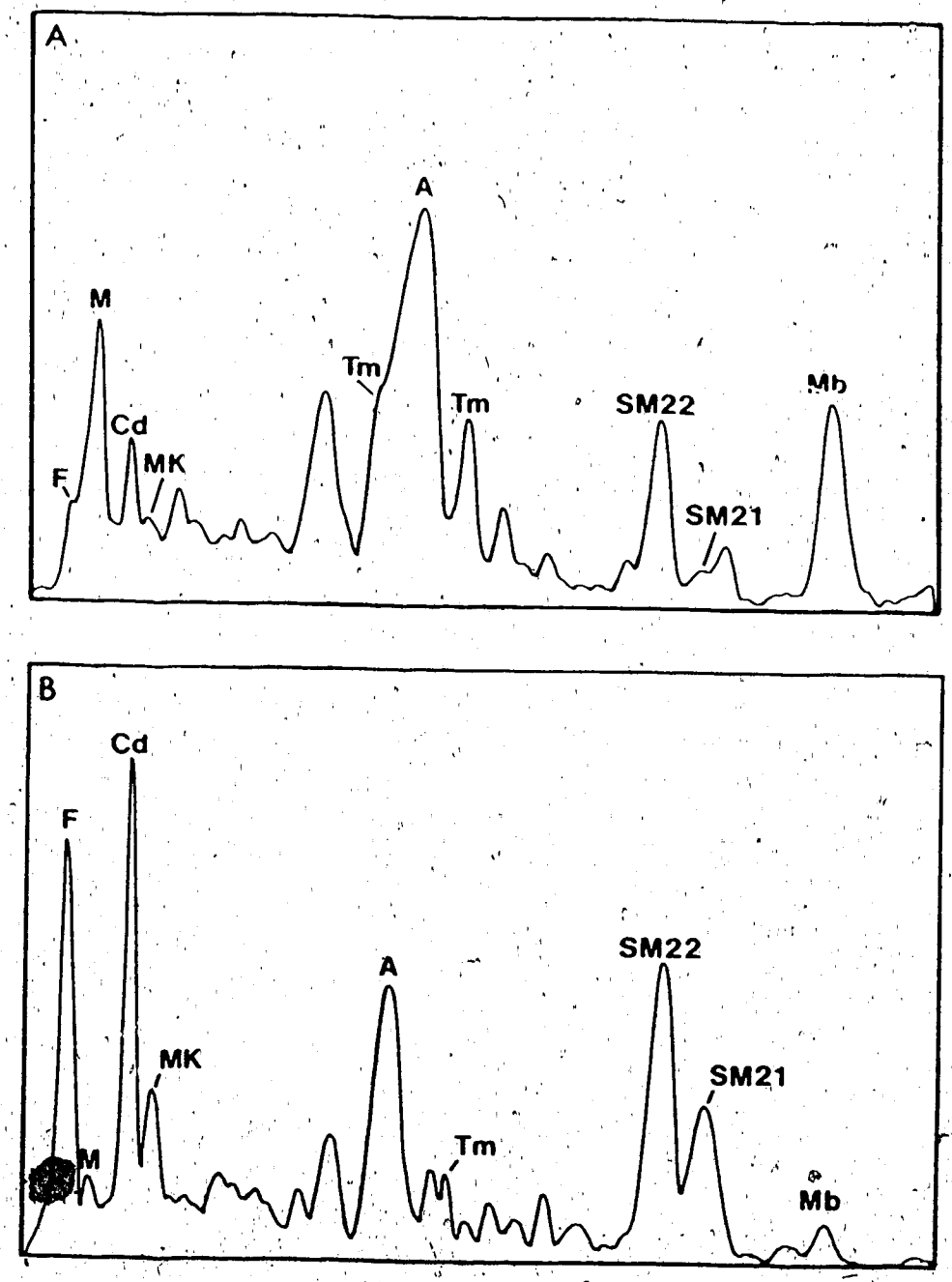


FIG. 14. Densitometric scans of chicken gizzard smooth muscle proteins. (A) SDS-urea extract of whole muscle corresponding to Fig. 13, lane a. (B) High Mg^{2+} extract of myofibrils corresponding to Fig. 13, lane e. Myosin, (M) filamin (F), caldesmon (Cd), myosin light chain kinase (MK), actin (A), tropomyosin (Tm), myoglobin (Mb).

TABLE II

Non-denaturing Purification of SM21 from Gizzard Smooth Muscle. Details of the Purification are Given in Materials and Methods.

Fraction	Total Protein	SM21	SM21	Yield
	g	% total protein	g	%
Whole smooth muscle SDS-urea extract	51	0.77	0.39	100
High Mg^{++} extract	1.7	12	0.21	54
Column chromatography	0.08	95	0.08	21

SM21 was quantitated by scanning densitometry of Coomassie Blue stained gels (see Figs. 13 and 14). These values were related to a standard curve of [SM21] versus peak area for identically stained gels.

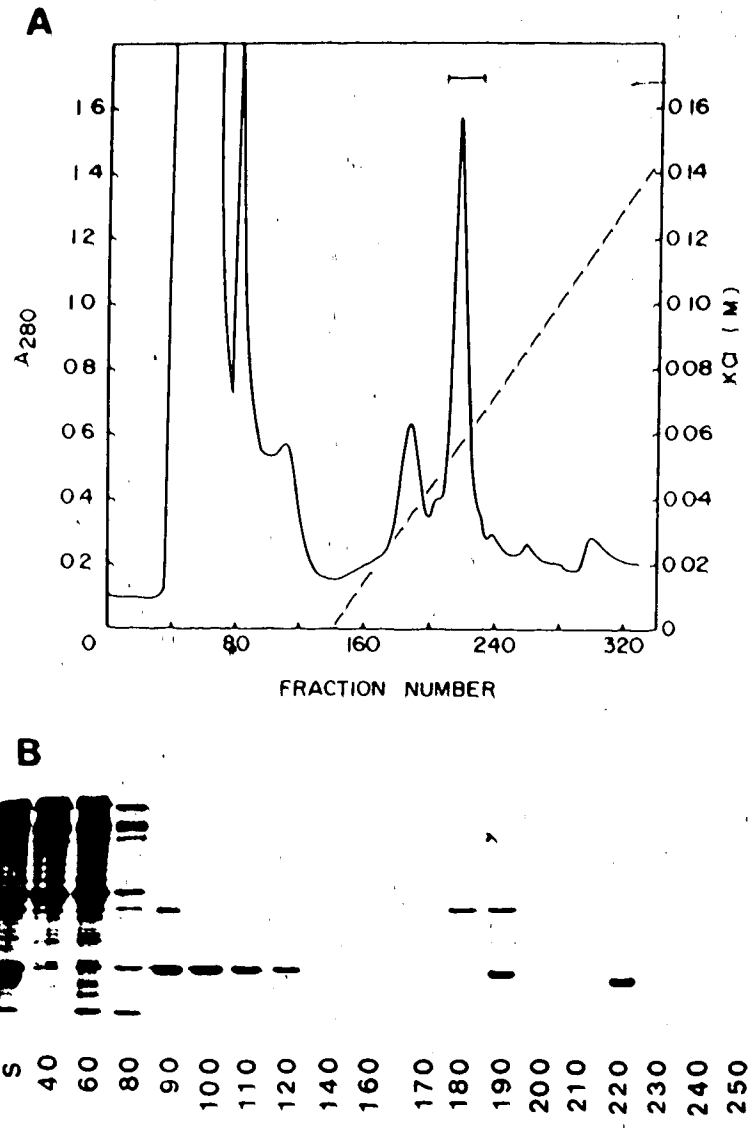


FIG. 15. CM-cellulose chromatographic purification of SM21 under non-denaturing conditions. (A) Chromatographic profile; the high Mg^{2+} extract which had been concentrated to 200 ml by Militan ultrafiltration and dialyzed as described in Materials and Methods was applied to a column (5 x 45 cm) previously equilibrated with buffer containing 50 mM sodium phosphate, pH 6.5 and 1 mM DTT. The column was then developed with 1.5 l of the same buffer, followed by a 2 l linear KCl gradient (0-0.15 M). Flow rate, 100 ml/h; fraction size, 10 ml; (⊢——⊣) material pooled for Affi-gel blue. (B) SDS-polyacrylamide gel electrophoresis of individual column fractions. (S) material loaded on column.

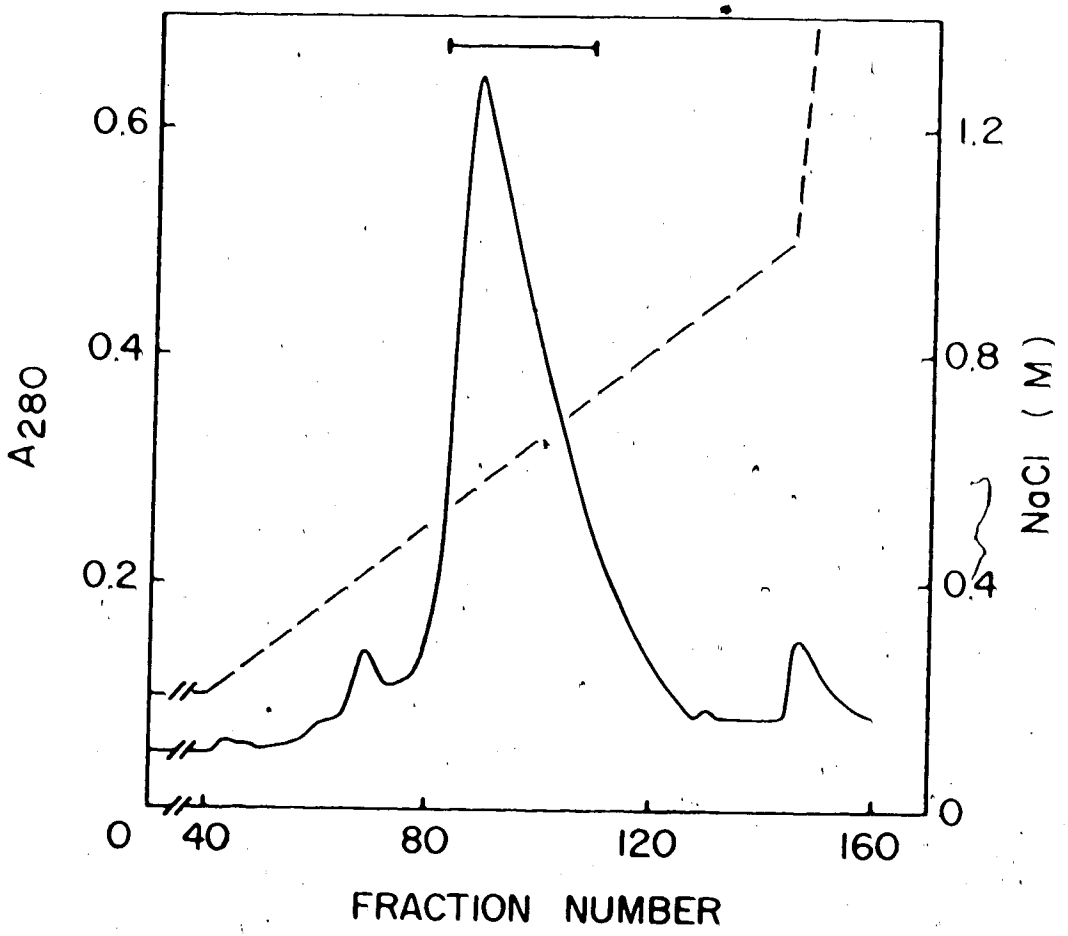


FIG. 16. Affi-gel Blue chromatographic purification of SM21. The pH of material pooled from CM-cellulose was adjusted to 7.0 and loaded on a column (2.5 x 15 cm) that had been previously equilibrated with a buffer containing 50 mM potassium phosphate, pH 7.0, 1 mM dithiothreitol. The column was developed with 200 ml of this buffer followed by 200 ml of the same buffer containing 0.2 M NaCl, followed by a 1 ℓ linear NaCl gradient (0.2 to 1.2 M). Flow rate, 50 ml/h, fraction size, 10 ml. (⟝⟞) indicates fractions pooled to obtain purified SM21 (see Fig. 13 A, lane f).

SM21 was concentrated using a Millipore CX-30 immersible filter and a Centricon 30 microconcentrator. It was found to be soluble to a concentration of at least 7 mg/ml in a solution containing 100 mM KCl, 50 mM sodium phosphate, pH 7.0 and 1 mM DTT. Amino acid analysis did not indicate any significant difference between SM21 purified under denaturing or non-denaturing conditions (Table I). NH_2-terminal amino acid analysis of carboxymethylated non-denatured SM21 also confirmed its identity with denatured SM21 (Fig. 5). In addition SM21 purified under non-denaturing conditions has the ability to bind ^{125}I-tropomyosin following electrophoresis on denaturing gels (Fig. 13 A to 13 C; lanes e and f).

Protein Binding Properties of SM21

The specificity of ^{125}I-tropomyosin binding to SM21 was tested by making several alterations in the binding solution (Fig. 13 E to 13 J ; lanes e and f). The control binding solution (5 ml) contained 0.15 M KCl, 0.05 M imidazole, pH 7.0, 1 mM DTT, and 0.15 μM ^{125}I-tropomyosin. Addition of 3 μM unlabeled tropomyosin resulted in a 75% loss of binding, 10 μM SM21 reduced binding by 60%, 0.5 M KCl reduced binding by 70% and replacement of binding solution by the high Mg^{2+} extraction buffer (see Methods) containing 0.15 μM ^{125}I-tropomyosin resulted in a 40% increase in binding.

In order to determine whether the binding of ^{125}I-tropomyosin to SM21 depended on prior denaturation of SM21, we conducted binding experiments using native gels (Fig. 17). It can be seen that SM22 does not bind ^{125}I-tropomyosin while SM21 does, thus showing the same relationship as on denaturing gels. SM21 purified under denaturing conditions did not enter the separating portion of the native gel but did bind ^{125}I-tropomyosin.

Calmodulin that had been ^{125}I-labeled with lactoperoxidase was also found to bind SM21 (Fig. 18). This binding was found to increase in the presence of 1 mM EGTA as compared to 1 mM $CaCl_2$. Myosin light chain kinase bound ^{125}I-calmodulin in the presence of 1 mM $CaCl_2$ but not in the presence of 1 mM EGTA as expected. Caldesmon was partially lost from the gel during the overlay procedure. What remained did not show ^{125}I-calmodulin binding activity.

In order to examine the possibility that SM21 bound to proteins other than tropomyosin and calmodulin, we labeled SM21 with ^{125}I using lactoperoxidase in a method similar to that for labeling tropomyosin. We found that ~~SM21 bound~~ primarily to actin in several chicken gizzard smooth muscle extracts that had been electrophoresed on denaturing gels (Fig. 19). It should be noted that tropomyosin is lost from the polyacrylamide gel during the course of the binding procedure (Fig. 6 A and 6B; lanes c and g).

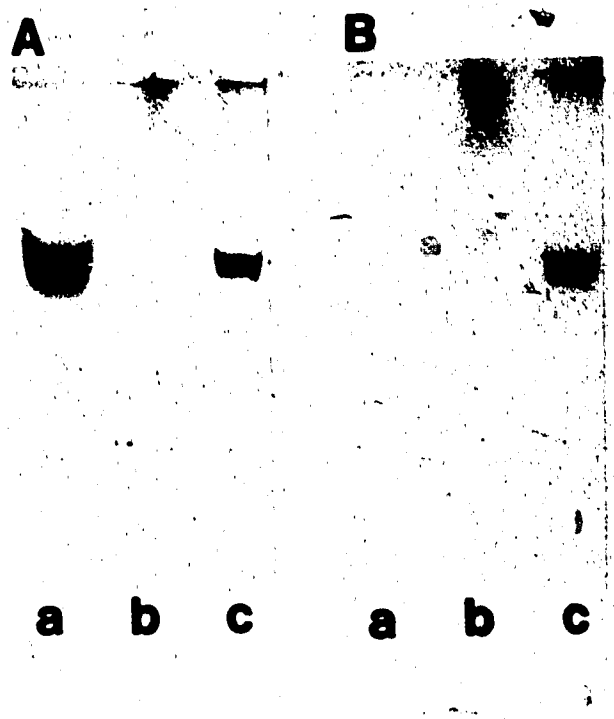


FIG. 17. Binding of ^{125}I-tropomyosin to SM21 on non-denaturing polyacrylamide gels. Non-denaturing gels were run according to the Pharmacia Guide to Polyacrylamide Gel Electrophoresis. The stacking gel was 5% polyacrylamide, 0.062 M acetic acid, pH 6.8 (KOH), the separating gel (5.5 cm in length, 0.15 mm in depth) consisted of 12% polyacrylamide, 0.375 M acetic acid, pH 4.3 (KOH) and the electrophoresis buffer was 0.35 M β-alanine, 0.14 M acetic acid, pH 4.5. (A) A non-denaturing gel that has been subjected to the ^{125}I-tropomyosin binding assay, with lanes (a) 4 μg of SM22, (b) 2 μg of SM21 purified under denaturing conditions, (c) 2 μg of SM21 purified under non-denaturing conditions. (B) Autoradiograph of I.

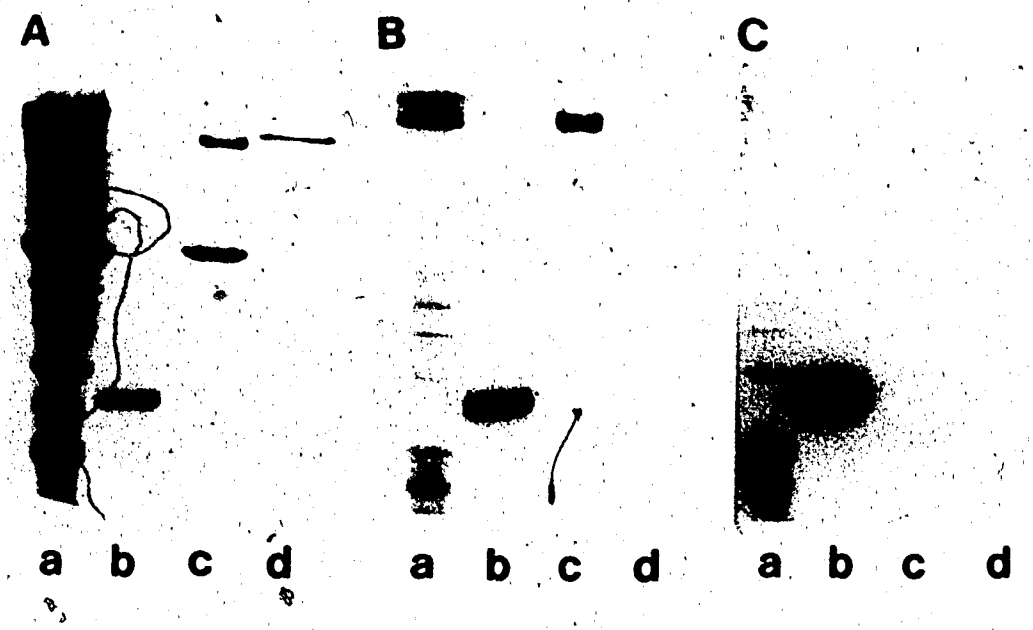


FIG. 18. Binding of ^{125}I-calmodulin to gizzard smooth muscle protein. Gel dimensions are length (9 cm), width (6 cm), depth (0.75 mm). (A) Coomassie blue stained gel with lanes (a) SDS-urea extract of chicken gizzard smooth muscle, (b) SM21 (4 μgm), (c) myosin light chain kinase (upper band) and actin, (d) caldesmon (2 μgm), (B) Autoradiograph of a gel similar to A that was incubated in the presence of ^{125}I-calmodulin (0.2 μM), 50 mM imidazole, pH 7.0, 0.15 M KCl, 1 mM dithiothreitol and 1 mM $CaCl_2$. (C) Autoradiograph of a gel incubated under conditions similar to B but with 1 mM EGTA replacing 1 mM $CaCl_2$.

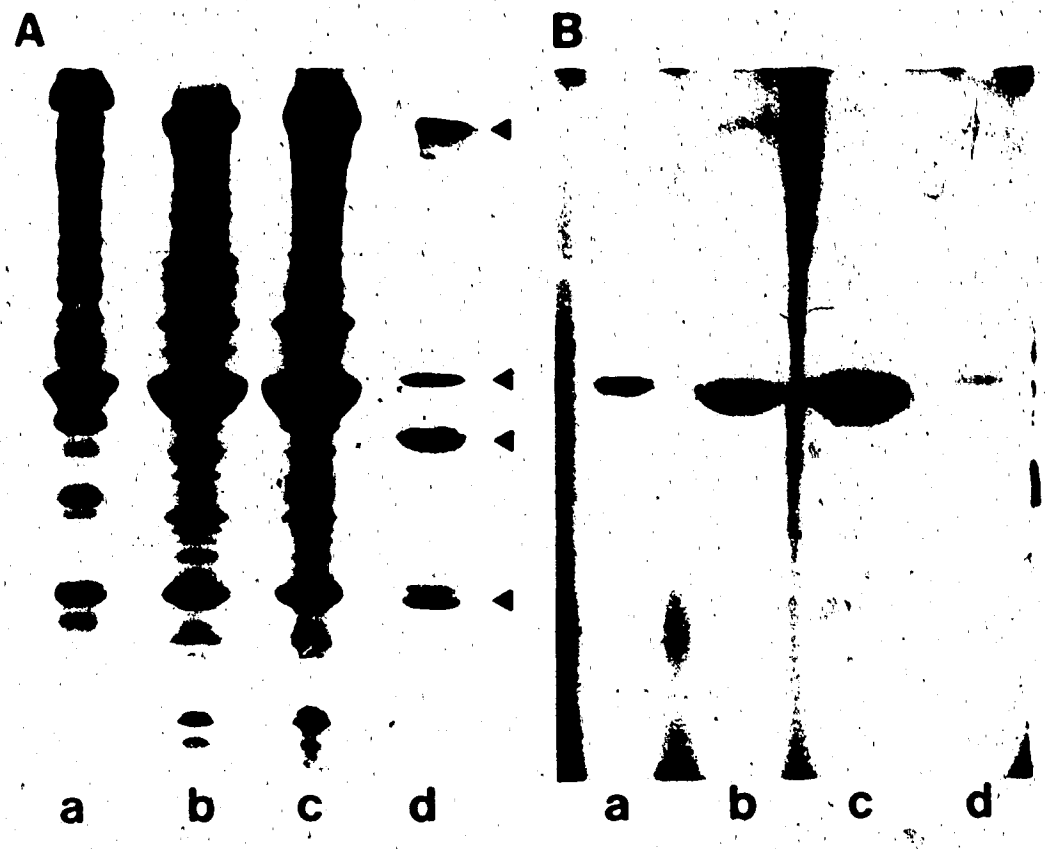


FIG. 19. Binding of ^{125}I-SM21 to gizzard smooth muscle extracts. The ^{125}I-SM21 binding assay was similar to the ^{125}I-tropomyosin binding assay (see Methods). (A) A gel with dimensions of length (9 cm), width (8 cm), and depth (0.75 mm) that has been subjected to the ^{125}I-SM21 binding assay. Lanes are (a) high Mg^{2+} extract of gizzard myofibrils, (b) low salt-Triton X-100 extract of gizzard smooth muscle, (c) SDS-urea extract of gizzard smooth muscle, (d) standards including from top to bottom, rabbit skeletal myosin, actin, troponin T and Troponin I. (B) Autoradiograph of A.

Physical Properties

The physical parameters determined for SM21 are listed in Table III. Sedimentation equilibrium experiments with SM21 yielded a straight line when the log of the protein concentration was plotted as a function of the square of the distance from the center of rotation (Fig. 20). The M_w of 21,200 calculated from the slope of this line is in good agreement with the value of 21,000 obtained from SDS-polyacrylamide gel electrophoresis. This agreement indicates that SM21 exists in the monomeric state at ionic strengths close to physiological. A plot of $s_{20,w}$ versus protein concentration (c) in mg/ml (Fig. 21) gave a regression line equation of:

$$s_{20,w} = 2.03 - 0.048c$$

Thus, SM21 behaves in a typical non-ideal manner and has an intrinsic sedimentation constant, $s^\circ_{20,w}$ of 2.03 S. In gel filtration experiments, SM21 produced a single symmetric peak with a Stokes radius ($R_{s,gel}$) of 21.8 Å (Fig. 22) while the calculated Stokes radius from sedimentation ($R_{s,sed}$) was 24.5 Å. A frictional ratio (f/f_{min} = 1.27) was determined for SM21 by dividing the average of $R_{s,gel}$ and $R_{s,sed}$ by the Stokes radius of an idealized spherical particle of identical M_w. When the estimated hydration of 0.41 g H_2O/g of protein is taken into account, the frictional ratio due to asymmetry alone (f/f_o) was found to be 1.09.

TABLE III

Physical Parameters of SM21

Parameter	Value
M_r sedimentation equilibrium	21,200
M_r SDS gel electrophoresis	21,000
Partial specific volume[a]	0.723 cm^3/g
$S^\circ_{20,w}$	2.03S
$R_{s,sed}$[b]	24.5 Å
$R_{s,gel}$[b]	21.8 Å
f/f_{min}[b]	1.27
hydration[c]	0.41
f/f_o[b]	1.11
$E^{1\ mg/ml}_{280}$[d]	1.22
$[\theta]_{205}$[e]	-8,600 degree $cm^2 dmol^{-1}$
$[\theta]_{220}$	-7,500 degree $cm^2 dmol^{-1}$
$\Delta\epsilon_{275}$[e]	-6.0 $M^{-1}cm^{-1}$

[a] Calculated from amino acid composition (Table I) as in (Cohn and Edsall, 1943).

[b] See General Materials and Methods for calculation methodology.

[c] Calculated from amino acid composition (Table I) as in (Kuntz and Kauzmann, 1974).

[d] Determined in 0.05 M sodium phosphate, pH 7.0 and 1 mM DTT.

[e] See Fig. 23.

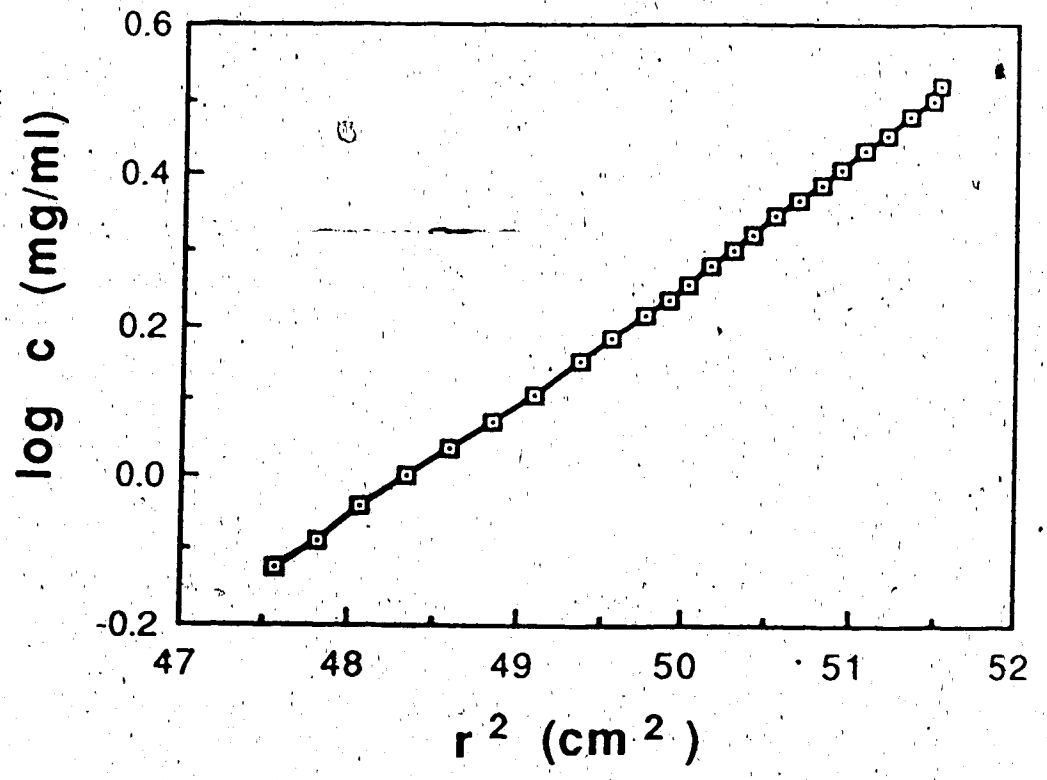


FIG. 20. Determination of the M_w of SM21 by sedimentation equilibrium. SM21 in 0.1 M KCl, 50 mM sodium phosphate, pH 7.0 and, 1 mM DTT was centrifuged at 17,000 rpm. $\bar{v}$ was calculated from amino acid composition as 0.723 and ρ was calculated as 1.01. Concentrations (c) were determined with Rayleigh interference optics.

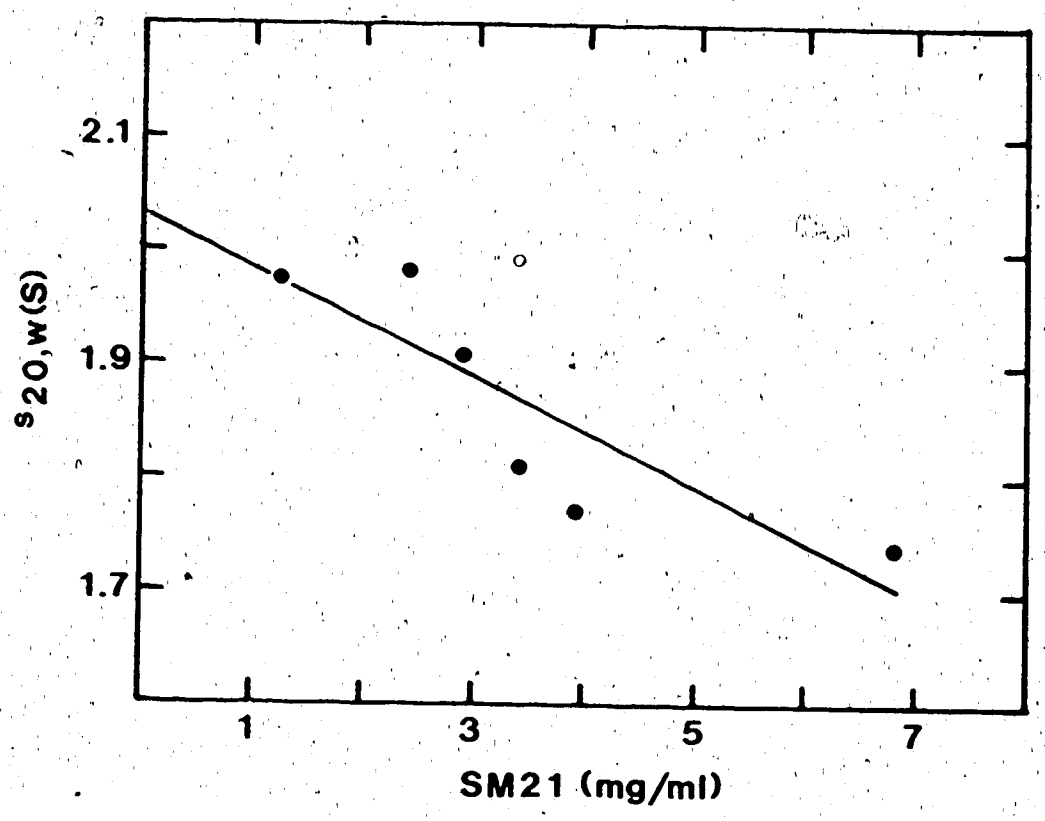


FIG. 21. Concentration dependence of the sedimentation coefficient of SM21. SM21 samples (0.4 ml) in 0.1 M KCl, 0.05 M sodium phosphate, pH 7.0, and 1 mM DTT were sedimented at 60,000 rpm using Kel F 2° centerpieces (12 mm) and schlieren photography.

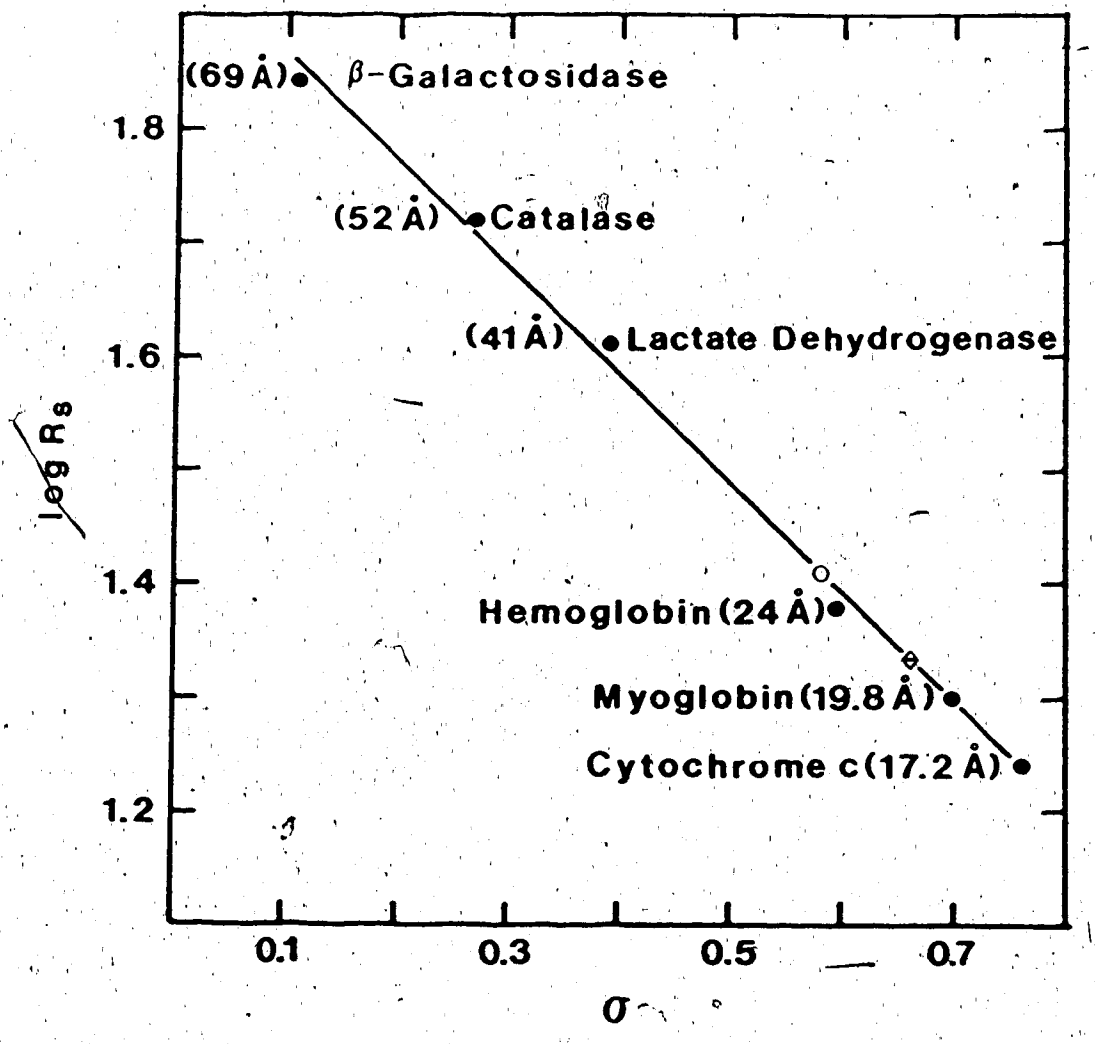


FIG. 22. Determination of the Stokes radius (R_s) of SM21 and SM22 by gel filtration. Samples (20 µl) in KMED buffer were applied to a 7.5 mm by 600 mm TSK G3000 SW column. Flow rate was 0.5 ml/min. Partition coefficients (δ) were calculated from peak elution volumes. SM21(θ) and SM22(o) were placed on the standard curve according to their partition coefficients.

Using a model for a prolate ellipsoid of revolution this value gives an axial ratio of 1:2.7, indicating that the monomeric SM21 molecule is moderately asymmetrical.

Circular Dichroism

The far-UV circular dichroic spectrum of SM21 is illustrated in Fig. 23. The greater dichroic intensity at 208 nm compared to 222 nm indicates that SM21 consists of separate α-helix and β-sheet rich regions (Manavalan and Johnson, 1983). We have used two sets of reference spectra to estimate the secondary structural content of SM21. The reference spectrum of Chen *et al.* (1974) which is based on the CD spectra of 5 proteins of known secondary structure (X-ray crystallography) yielded a structural content of 27% α-helix, 15% β-sheet and 58% random coil. When the CD spectrum of SM21 was analyzed according to a reference spectrum based on 16 proteins of known secondary structure (Provencher and Glockner, 1981) a composition of 17% α-helix, 32% β-sheet, 14% β-turn and 37% random coil was found.

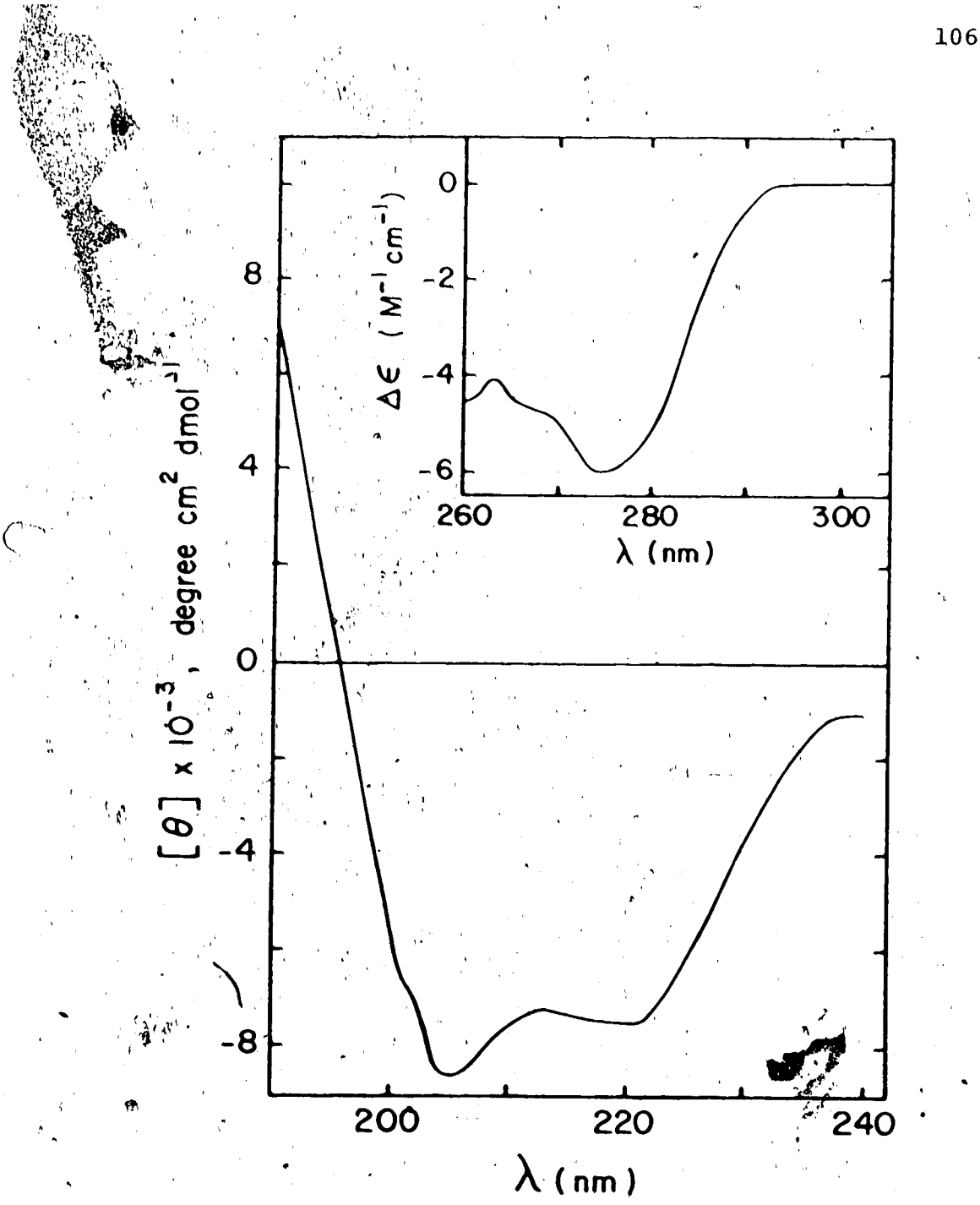


FIG. 23: Circular dichroic spectra of SM21. Solutions for CD contained 0.63 mg/ml SM21, 50 mM sodium phosphate (pH 7.0), 1.0 mM dithiothreitol. The path length was 0.0103 cm for the far ultraviolet and 1.0 cm for the near ultraviolet spectra. $\Delta\epsilon = \epsilon_L - \epsilon_R = [\theta]N/3300$ where ϵ_L and ϵ_R are the molar extinction coefficients for left and right circularly polarized light, [θ] is the mean residue ellipticity and N is the number of amino acid residues in SM21 (Strickland, 1974).

The near UV circular dichroic spectrum of SM21 (Fig. 23) consists primarily of a broad negative band that reaches a minimum at 275 nm. The intensity of this minimum ($\Delta\epsilon$ = 6.0) indicates that interactions involving aromatic residues occur within the structure of SM21 (Strickland, 1974). The spectrum is notable for the absence of tryptophan structure at 290 nm. This might indicate that the 2 tryptophans of SM21 are motile or that their spectra cancel. If the former case is correct, it is likely that the broad minimum at 275 nm results primarily from the estimated 10 tyrosine residues in SM21 (Table 1).

D. Discussion

We have modified the procedure of Ngai *et al.* (1984) for the purification of MLCK and caldesmon in order to isolate SM21 in its native form and in high yield. We found that a high Mg^{2+} extract of gizzard myofibrils resulted in a 16 fold increase in purity while retaining 60% of SM21. Proteins co-extracted in abundance with SM21 included several microfilament associated proteins such as filamin, caldesmon, MLCK, and actin, as well as SM22 which is characterized in chapter 5. This extract, however, contained only a small amount of tropomyosin. Salt precipitation of SM21 was avoided by employing a Militan ultrafiltration device to concentrate the high Mg^{2+} extract. This was followed by column chromatography at pH 6.5 (CM-cellulose) and pH 7.0 (Affi-gel Blue). The resulting SM21 was soluble to at least 7 mg/ml at physiological salt concentration. It was, however, indistinguishable from SM21 purified in the presence of 8 M urea, in terms of amino acid composition, N-terminal amino acid sequence, and binding to tropomyosin after electrophoresis on denaturing gels.

As mentioned above, tropomyosin is a minor component of the high Mg^{2+} extract. To see if SM21 would bind to tropomyosin under similar conditions, we replaced our normal binding assay buffer with the high Mg^{2+} extract buffer. Under these conditions, SM21 bound 40% more ^{125}I-tropomyosin. Thus a factor other than buffer composition must be responsible for the lack of co-extraction of SM21 and

tropomyosin in the high Mg^{2+} buffer.

In order to investigate the possibility that interaction of SM21 with proteins other than tropomyosin is responsible for this lack of co-extraction, we labeled SM21 with lactoperoxidase and studied its binding to gizzard extracts on denaturing gels. We found that the main binding protein in these extracts, including a high Mg^{2+} extract was actin. It is therefore possible that during the high Mg^{2+} extraction, SM21 is being co-extracted with actin rather than remaining bound to tropomyosin. It should be noted that tropomyosin is lost from polyacrylamide gels during the overlay procedure (see Fig. 6 A and 6 B lane e and g). We also found that ^{125}I-calmodulin will bind to SM21 on denaturing gels. This binding was stronger in the presence of EGTA than Ca^{2+}.

It is also possible that tropomyosin will bind to SM21 only after SM21 has been electrophoresed on a denaturing gel. We therefore tested SM21's ability to bind to a tropomyosin-Sepharose 4B affinity column. While this column did bind the T2 fragment of troponin-T, it did not bind SM21 (unpublished results). However, when SM21 was electrophoresed on a native gel, it still bound ^{125}I-labelled tropomyosin. Thus, while electrophoresis of SM21 on polyacrylamide gels appears to be an important factor in its binding to tropomyosin, prior denaturation does not appear to be essential. It is also possible that SM21 is denatured at the pH of the native gel (pH 4.3).

We also examined some of the conditions that affect ^{125}I-tropomyosin binding to SM21 on denaturing gels. We found that a 20-fold excess of unlabeled tropomyosin (3 μM) reduced binding of ^{125}I-tropomyoson by only 75%, thus it appears that binding of tropomyosin to SM21 is not saturated at 0.15 μM tropomyosin. Addition of 2 mg of SM21 to 5 ml of binding solution inhibited ^{125}I-tropomyosin binding to 2 μg of gel localized SM21 by only 60%, indicating that gel localized SM21 has greater affinity for tropomyosin than does SM21 in solution. This correlates with the finding that SM21 does not bind to a tropomyosin affinity column. Addition of 0.5 M NaCl to the binding solution reduces SM21-tropomyosin interaction by 70% which points to the presence of an ionic component in this interaction.

Physical characterization of SM21 indicated that it is a moderately asymmetrical (globular) monomer at physiological salt and pH 7.0. Analysis of the far-UV circular dichroic spectrum of SM21 indicates that it contains a relatively high proportion of random coil. There was, however, a considerable difference between the structural content prediction of the two reference spectra that were employed. This difference may result from inadequate representation of proteins with structures similar to SM21 in one or both of the databases. It has recently been pointed out that the structural portion of proteins referred to as random coil is not actually random. Rather, much of this structure forms loops that may be important in

protein-protein and co-factor-protein interactions (Leszczynski and Rose, 1986). The possible presence of multiple binding loops in SM21 as represented by its high content of random coil might help to explain its diversity of protein-protein interactions.

To summarize, we have purified a 21 kDa tropomyosin binding protein from chicken gizzard smooth muscle. While we have not proved that this binding is physiologically significant, the persistant presence of SM21 in extracts of contractile apparatus associated proteins indicates that it may play an important role in the structure and function of this apparatus. In addition, we have used amino acid analyses and sequencing to demonstrate that SM21 is not similar to troponin-T or troponin-I, but is a unique protein that is present in such diverse smooth muscle containing organs as chicken gizzard and bovine aorta.

I would also like to mention at this point the possible usefullness of the myofibril preparation for the purification of 4 other high mobility basic proteins (SM32, SM24, SM22, and SM20) from smooth muscle (see Fig. 1.1). Each protein behaves differently during this preparation. SM32 remains largely in the material not extracted by low salt or high Mg^{2+}. The low salt washes extract a large amount of SM24 and SM22 but leave a fair amount for extraction with high Mg^{2+}. SM20 along with myoglobin is almost completely extracted by the low salt washes. Purification of SM22 and SM20 will be described in the following chapters.

V. Purification and Characterization of an Abundant and Novel 22 kDa Protein (SM22)

A. Introduction

In our continuing effort to find troponin like components in smooth muscle we have purified a highly abundant, basic, 22 kDa protein (SM22). This protein was seen in chapter 3 to be present in the distilled water precipitate along with SM21. It was also found in the high Mg^{2+} extract of chapter 4, but it was present in greatest amounts in the distilled water supernatant of chapter 3 and its purification from this source is described in this chapter. While SM22 can be seen on the gels of others (Kendrick-Jones, 1973 ; Sobieszek and Bremel, 1975 ; Hirai and Hirabayashi, 1983 ; Walsh *et al.*, 1983 ; Adachi *et al.*, 1983 ; Ngai *et al.*, 1984 ; Murakami and Uchida, 1985) its isolation and characterization have not previously been reported. In the present chapter we describe a method for purification of SM22 in good yields, as well as a number of its chemical and physical properties. Based on amino acid sequence (Pearlstone *et al.*, 1987), amino acid composition and other characteristics, SM22 appears to be unlike any previously characterized protein.

B. Materials and Methods

Fresh chicken gizzards that had been cut open and washed were donated by Lilydale Poultry Packers (Edmonton). Smooth muscle was cut away from the tough inner lining and most of the external connective tissue sheath. All further purification steps were carried out at 4°C. Muscle (2 x 250 g) was homogenized in 2 x 1.75 l of extraction buffer (1 M KCl, 0.05 M imidazole, pH 7.0, 1 mM EGTA, 0.25 mM phenylmethylsulfonyl fluoride, 100 μg/ml soybean trypsin inhibitor, 0.2 μg/ml leupeptin, 0.2 μg/ml pepstatin, and 1 mM DTT) in a 3 l Waring blender. Blending was done at high speed in three, 15 s runs spaced by 3 min intervals. The homogenate was centrifuged at 16,000 x *g* for 60 min. The resulting supernatant was filtered through glass wool and dialysed against 2 x 15 l of 0.02% mercaptoethanol for 2 x 7 h. After clarification by centrifugation at 16,000 x *g* for 45 min, the supernatant was adjusted to pH 4.7, stirred for 15 min and centrifuged (16,000 x *g*; 45 min). Following adjustment of the supernatant to pH 7.0, an ammonium sulfate fraction from 35-70% saturation was collected (16,000 x *g*; 45 min), resuspended in 0.2 l of 0.02% mercaptoethanol, dialysed against the same solution and lyophilized. The lyophilized protein was then dissolved in 200 ml of column buffer and fractionated by CM-cellulose (Whatman-32) column chromatography (see Fig. 27). The salt concentration of the column eluate was monitored with a Radiometer conductivity meter. For determination of yields at each step of the

purification, a sample of each fraction was electrophoresed on SDS-polyacrylamide gels and stained with Coomassie Blue. The relative proportion of SM22 to total protein was then estimated by gel scanning.

Electrophoresis

SDS-polyacrylamide gel electrophoresis was run using a Laemmli (1970) buffer system with a 5% stacking gel. The positions of filamin, myosin, caldesmon, α-actinin, desmin, actin, tropomyosin and myoglobin on gels was determined according to relative abundances and apparent M_r as described by Hirai and Hirabayashi (1983), Feramisco and Burridge (1980) and Ngai and Walsh (1985b). Histones were purified from the distilled H_2O precipitate (Fig. 26, lane 5) and albumin from the first peak of the CM-cellulose chromatographic column (see Fig. 27) (unpublished work).

C. Results

SDS-polyacrylamide gel electrophoresis of a whole gizzard smooth muscle extract demonstrates the presence of a major protein band at 22 to 24 kDa (Fig. 24). Densitometric scanning indicated that the approximate molar ratio of actin: SM22: tropomyosin in this extract is 6.5 ± 0.8: 2.0 ± 0.2: 1.0. When a urea extract of gizzard smooth muscle is electrophoresed on a two-dimensional gel, SM22 can be identified as a basic protein with an isoelectric point similar to that of myoglobin (Fig. 25). It is present as 3 isoelectric variants in ratios of 14(α): 5(β): 1(γ).

Purification of SM22

We have succeeded in developing a rapid high yield method for purification of SM22α and SM22β. A brief summary of the purification is presented in Table IV. The first step is extraction with 1 M KCl (Fig. 26, lane 3). Dialysis of the 1 M KCl extract against distilled H_2O quantitatively precipitates histones, desmin, myosin and caldesmon. It also precipitates some actin, tropomyosin and α-actinin and about 20% of SM22 (Fig. 26,, lanes 4 and 5). Acidifying the distilled H_2O supernatant to pH 4.7 removes the majority of remaining actin, tropomyosin and filamin (Fig. 26, lanes 6 and 7). $(NH_4)_2SO_4$ fractionation was then used to precipitate SM22 from the pH 4.7 supernatant and remove myoglobin, which remains in the 70% supernatant (Fig. 26 lanes 8-11).

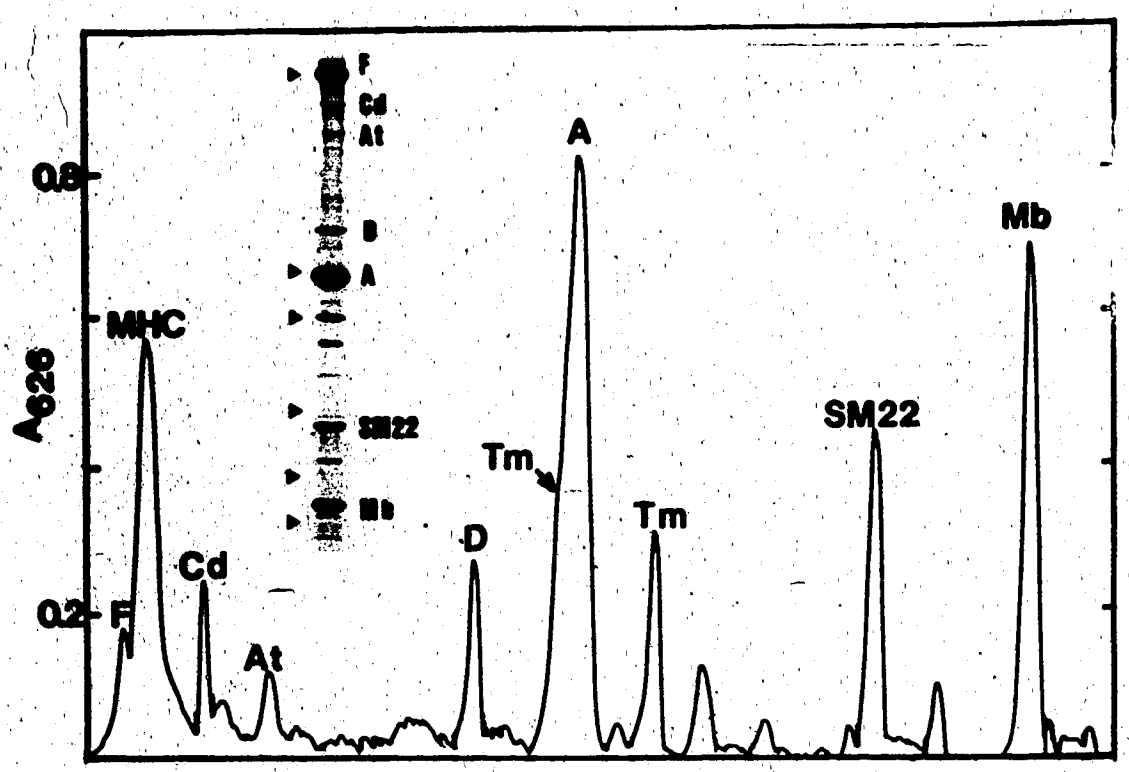


FIG. 24. Densitometric scan of chicken gizzard smooth muscle proteins from a 10-18% gradient SDS-polyacrylamide gel. Filamin (F), myosin heavy chain (MHC), caldesmon (Cd), α-actinin (At), desmin (D), actin (A), tropomyosin (Tm), myoglobin (Mb). The inset is a photograph of the gel that was scanned above. M_r markers (arrowheads) include from top to bottom, rabbit skeletal myosin heavy chain (200,000), gizzard tropomyosin subunits (43,000 and 38,000) and rabbit skeletal myosin light chains (25,000, 18,000 and 15,000).

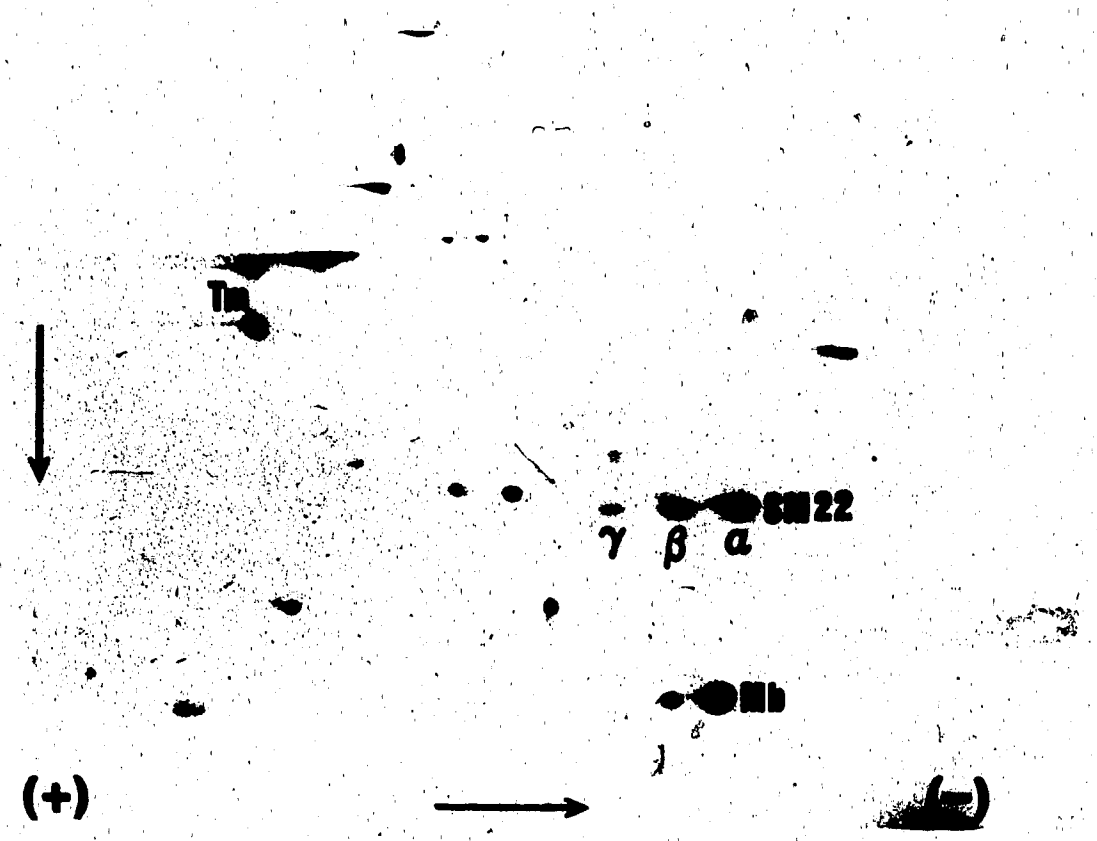


FIG. 25. Two-dimensional gel of a 9 M urea extract of gizzard smooth muscle. The horizontal dimension is non-equilibrium isoelectric focusing carried out for 3000 Vh in the presence of pH 3-10 ampholines (LKB). The vertical dimension is SDS-polyacrylamide gel electrophoresis. The variants of SM22 are marked as α, β, γ.

TABLE IV

Purification of SM22 from gizzard smooth muscle

Details of the purification procedure are described under Materials & Methods

Fraction	Total Protein	SM22	SM22	Yield
	g	% total protein	g	%
Whole smooth muscle SDS-urea extract	56	4.5	2.5	100
1 M KCL extract	24	7.3	1.75	70
Distilled H_2O supernatant	13	10	1.3	52
pH = 4.7 supernatant	4.2	29	1.2	48
70% $(NH_4)_2SO_4$ precipitate	2.2	41	0.9	36
CM-cellulose	0.6	> 95	0.6	24

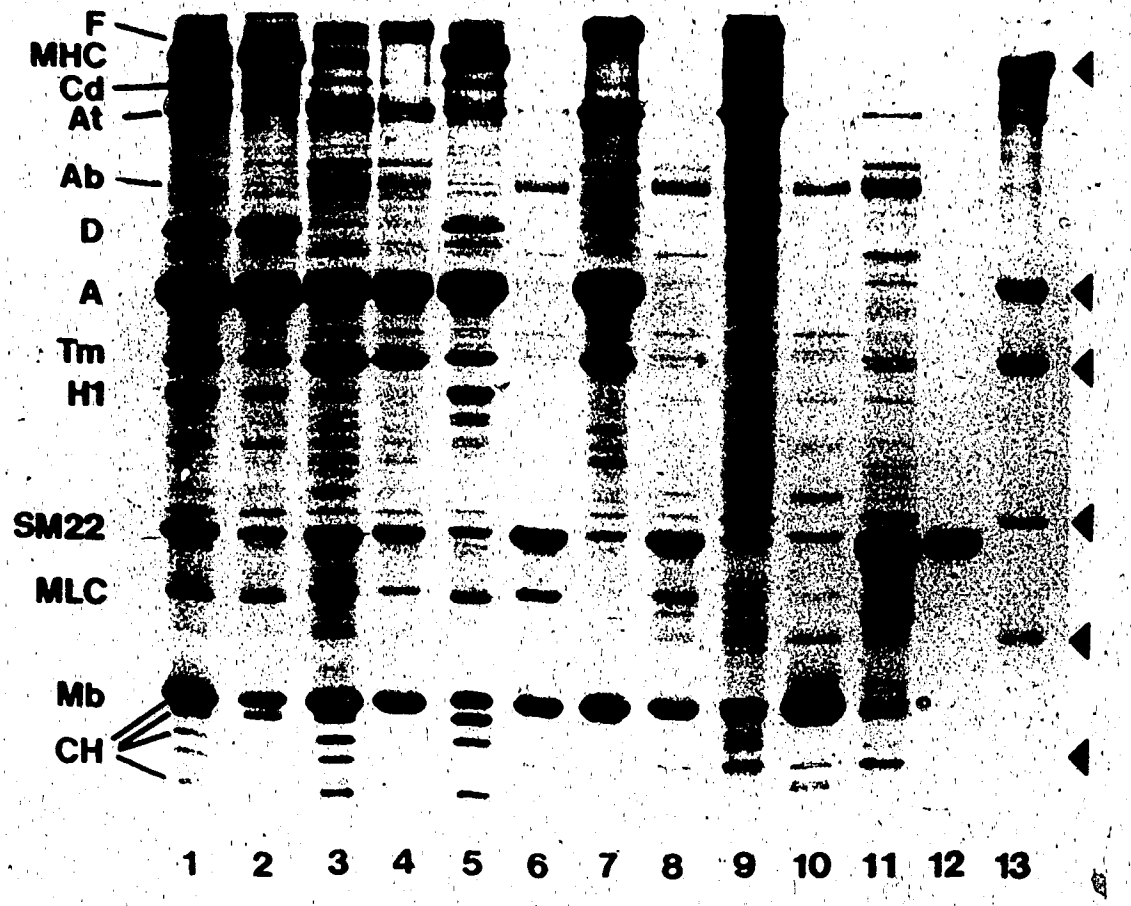


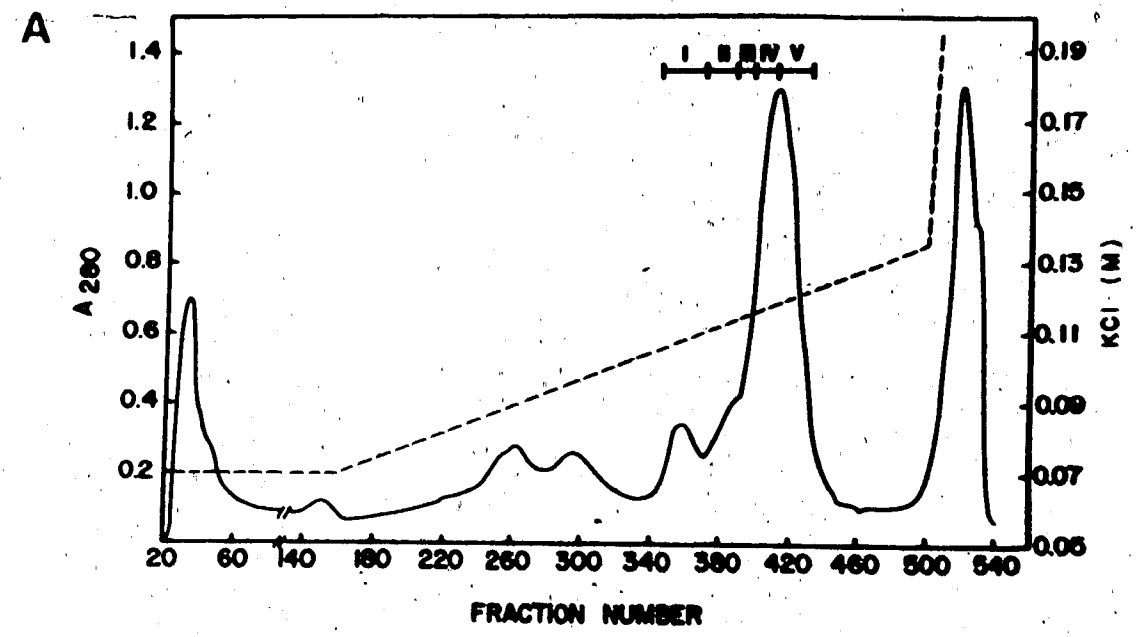
FIG. 26. SDS-polyacrylamide gel electrophoresis of fractions at various stages of SM22 purification. Lanes are (1) SDS-urea extract of gizzard, (2) material insoluble in 1 M KCl, (3) 1 M KCl extract, (4) distilled H_2O supernatant, (5) distilled H_2O precipitate, (6) pH 4.7 supernatant, (7) pH 4.7 precipitate, (8) 35% $(NH_4)_2SO_4$ supernatant, (9) 35% NH_4SO_4 precipitate, (10) 70% $(NH_4)_2SO_4$ supernatant, (11) 70% $(NH_4)_2SO_4$ precipitate, (12) purified SM22, (13) M_r standards as in Fig. 1. Abbreviations are as in Fig. 1 and 2, with the addition of histone H1 (H1), the core histones (CH) (from top to bottom H3, H2B, H2A, H4) and the 20 kDa myosin light chain (MLC).

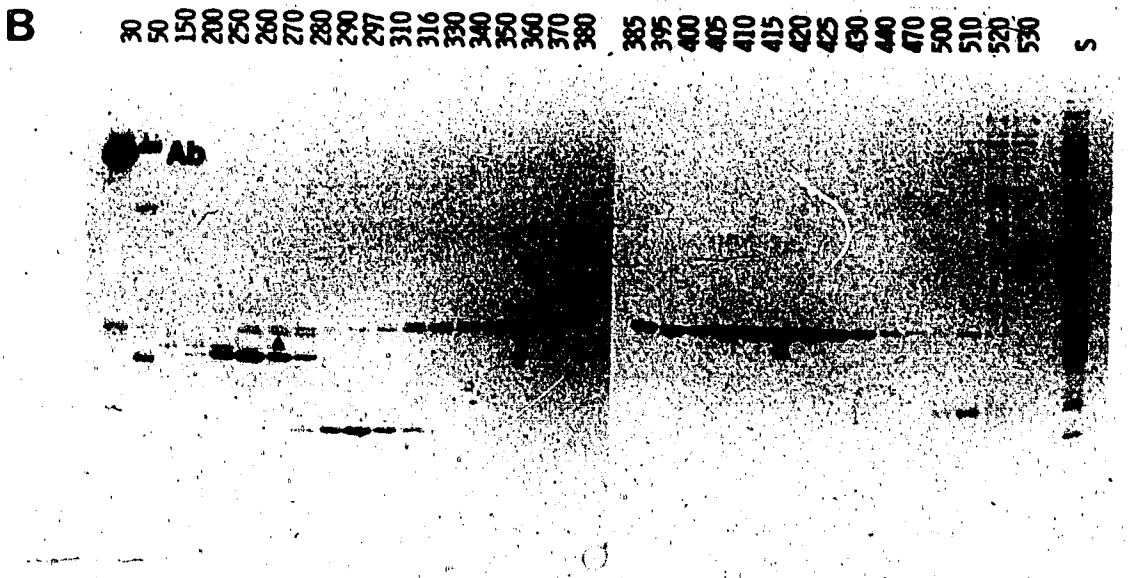
Final purification of SM22 and separation of its variants was carried out by CM-cellulose column chromatography (Fig. 27 A). SDS-Polyacrylamide gel electrophoresis of the column fractions illustrates the separation of components α, β and γ (Fig. 27 B). A shoulder on the leading edge of the major peak in Fig. 27 A indicates the possible presence of a fourth variant. Indeed 2-dimensional gel electrophoresis of the shoulder fraction indicates that it has an apparent M_r equal to α but an isoelectric point equal to β (Fig. 27 C). We were unable to resolve this variant (β') on 2-dimensional gels of whole gizzard extracts (Fig. 25). This may be due to a lower resolution obtainable with whole extracts or it could indicate that β' is an artifactual modification produced during purification.

Amino Acid Composition and Sequencing

Amino acid analyses of SM22α and β (Table V) indicate that they are highly similar in composition. The major difference is the presence of one half-cystine in α and two half-cystines in β. β also appears to have fewer basic residues which might account for its more acidic isoelectric point. Table V compares the amino compositions of SM22 with myokinase (Van Zabern *et al.*, 1976), skeletal troponin I (Wilkinson and Grand 1978), and a cytosolic 23 kDa basic protein from brain (Bernier and Jollès, 1984).

FIG. 27. CM-Cellulose Chromatographic Purification of SM22. (A) Chromatographic Profile; 2 g of lyophilized 70% $(NH_4)_2SO_4$ precipitate in 100 ml of buffer (70 mM NaCl, 50 mM sodium acetate, 1 mM EGTA, pH 4.7) were applied to the column (5 x 45 cm) previously equilibrated with the same buffer. The column was then developed with 1.6 liter of the same buffer followed by a 3 liter linear NaCl gradient (70 to 125 mM). Flow rate; 100 ml/h; fraction size, 9 ml. (B) SDS-polyacrylamide gel electrophoresis of individual fractions. Loadings for fractions 30 to 380 were four times those of 385 to 530 to permit visualization of SM22γ. (C) Two-dimensional gels of fractions I to V from A above. Sperm whale myoglobin (3-5 μg) (see arrowheads) was added to each fraction to provide a reference for comparison of SM22 isoforms α, β, β', and γ.


A
I II III IV V
A 280
KCl (M)
FRACTION NUMBER
20 60 140 180 220 260 300 340 380 420 460 500 540
1.4 1.2 1.0 0.8 0.6 0.4 0.2
0.19 0.17 0.15 0.13 0.11 0.09 0.07 0.05


B
30 50 150 200 250 260 270 280 290 297 310 316 330 340 350 360 370 380 385 395 400 405 410 415 420 425 430 440 470 500 510 520 530 S
Ab

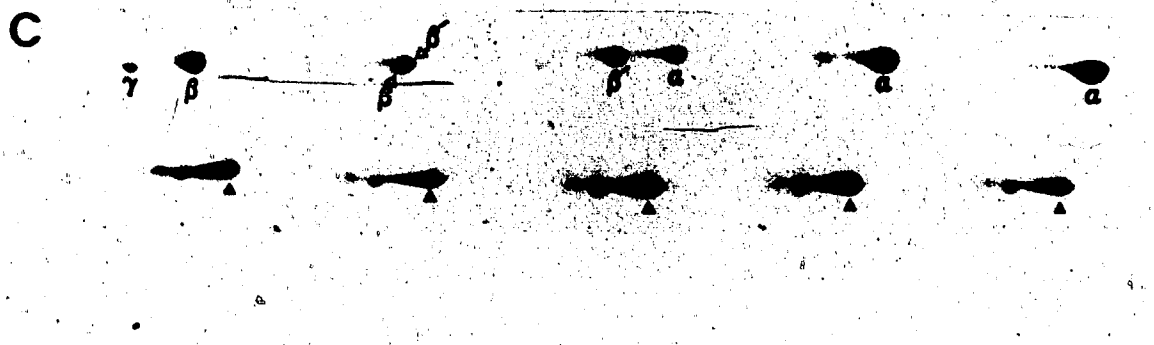

C
γ
β
α

TABLE V

Amino acid compositions of SM22 variants and other basic proteins with similar M_r

The composition of SM22 α and β were based on 14 alanines in order to approach the sedimentation equilibrium molecular weight of SM22 α and keep the number of histidines near an integral value. Samples of SM22 were hydrolysed for 24, 72 and 120 hrs. Values for serine and threonine were extrapolated to 0 time. Valine and isoleucine were taken from the average of 72 and 120 hr hydrolysates. M_r is calculated from the amino acid composition.

Residue	SM22α	SM22β	Myokinase[a]	Brain 23 kDa[b] protein	Troponin-I[c]
Asx	19	19	12	22	15
Thr	6.2	6.8	14	10	3
Ser	13	13	11	17	10
Glx	25	24	27	20	25
Pro	9.5	9.6	6	17	5
Gly	18	17	19	19	8
Ala	14	14	8	8	14
Val	14	14	17	15	7
Met	7.5	6.8	5	2	9
Ile	6.5	6.5	9	4	5
Leu	14	14	18	21	17
Tyr	6.0	5.8	7	10	2
Phe	7.1	6.9	5	6	3
His	3.2	3.3	2	6	4
Lys	18	17	19	16	24
Arg	9.2	8.4	13	10	15
Trp	3.0	2.9	0	5	1
Half-Cys	1.1	2.2	2	2	3
Total	195	192	194	210	178
M_r	21,700	21,400	21,700	23,500	20,700

[a] From amino acid sequence of human muscle isoenzyme I adenylate kinase; Van Zabern et al., (1976)

[b] See Bernier and Jolles (1984)

[c] From amino acid sequence of rabbit skeletal troponin-I; Wilkinson and Grand (1978)

While these proteins have similar sizes and isoelectric points, their compositions do not indicate homology with SM22. Attempts to sequence intact SM22 from its NH_2-terminus were not successful indicating that it was blocked.

Physical Properties

Physical parameters determined for SM22α are listed in Table VI. Sedimentation equilibrium experiments with SM22α yielded a straight line when the log of protein concentration was plotted as a function of the square of the distance from the center of rotation (Fig. 28). The M_r of 22,000 calculated from the slope of this line is in good agreement with the value of 22,000 to 24,000 obtained from SDS-polyacrylamide gel electrophoresis. This agreement indicates that SM22α exists in the monomeric state at ionic strengths close to physiological. A plot of $s_{20,w}$ versus protein concentration (c) (Fig. 29) gave a regression line equation of:

$$s_{20,w} = 2.01 - 0.031c$$

Thus, SM22 behaves in a typical non ideal manner and has an intrinsic sedimentation constant, $s^{\circ}_{20,w}$ of 2.01 S. In gel filtration experiments (Fig. 22), SM22α produced a single symmetric peak with a Stokes radius ($R_{s,gel}$) of 25.8 Å, which agrees well with a calculated Stokes radius from

TABLE VI

Physical Parameters of SM22a

Parameter	Value
M_r sedimentation equilibrium	22,000
M_r SDS gel electrophoresis	23,000
Partial specific volume[a]	0.734 cm^3/g
$S^\circ_{20,w}$	2.01 S
$R_{s,sed}$[b]	25.1 Å
$R_{s,gel}$[b]	25.8 Å
f/f_{min}[b]	1.35
hydration[c]	0.38
f/f_o[b]	1.18
$E^{1\ mg/ml}_{280}$[d]	1.17
$[\theta]_{206}$[e]	-18,900 degree $cm^2 dmol^{-1}$
$[\theta]_{219}$	-14,100 degree $cm^2 dmol^{-1}$
$[\theta]_{189}$	28,800 degree $cm^2 dmol^{-1}$
$\Delta\epsilon_{293}$[e]	3.6 $M^{-1}\ cm^{-1}$
$\Delta\epsilon_{287}$	-1.3 $M^{-1}\ cm^{-1}$
$\Delta\epsilon_{280}$	-2.2 $M^{-1}\ cm^{-1}$

[a] Calculated from amino acid composition (Table II) Cohn and Edsall (1943)

[b] See General Materials and Methods for calculation methodology

[c] Calculated from amino acid composition (Table II) Kuntz and Kauzmann (1974)

[d] Determined in 0.01% trifluoroacetic acid

[e] See Fig. 30

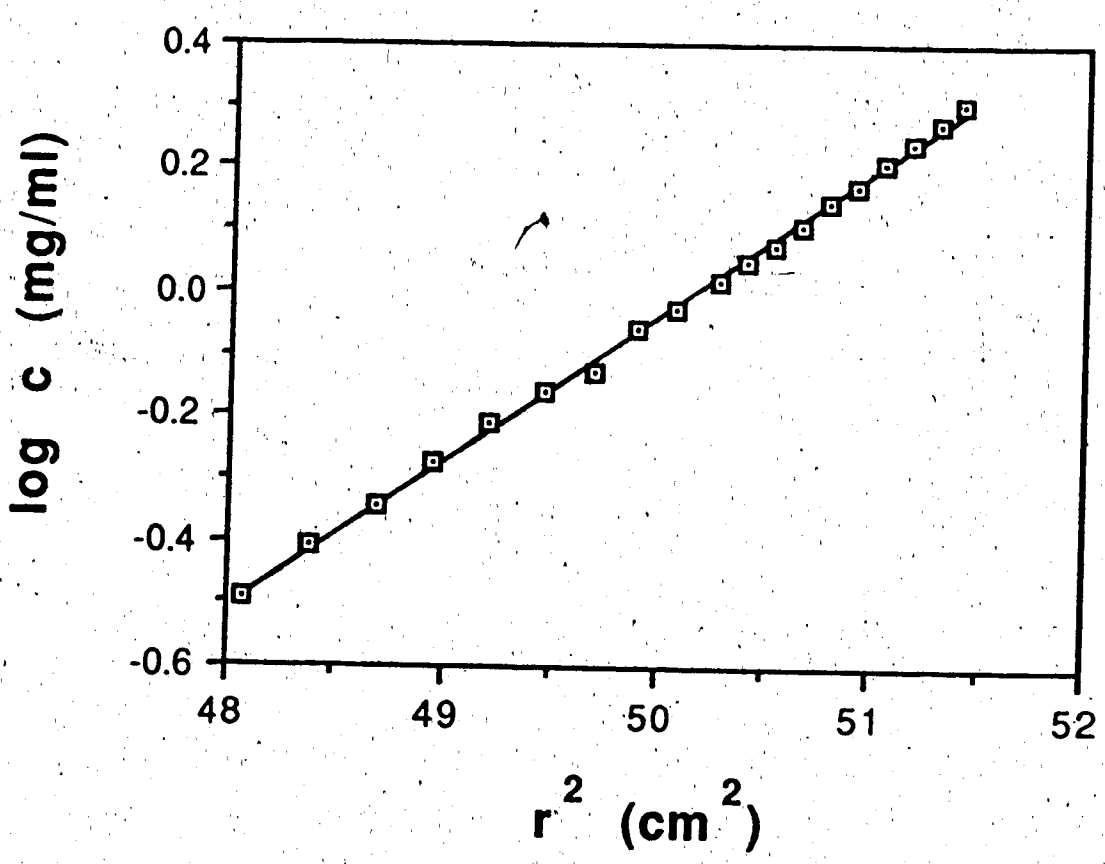


FIG. 28. Determination of the M_w of SM22 by sedimentation equilibrium. SM22 in KMED (0.1 ml) was centrifuged at 20,000 rpm. $\bar{v}$ was calculated from amino acid composition as 0.734 and ρ was calculated as 1.0045. Concentrations (c) were determined with Rayleigh interference optics.

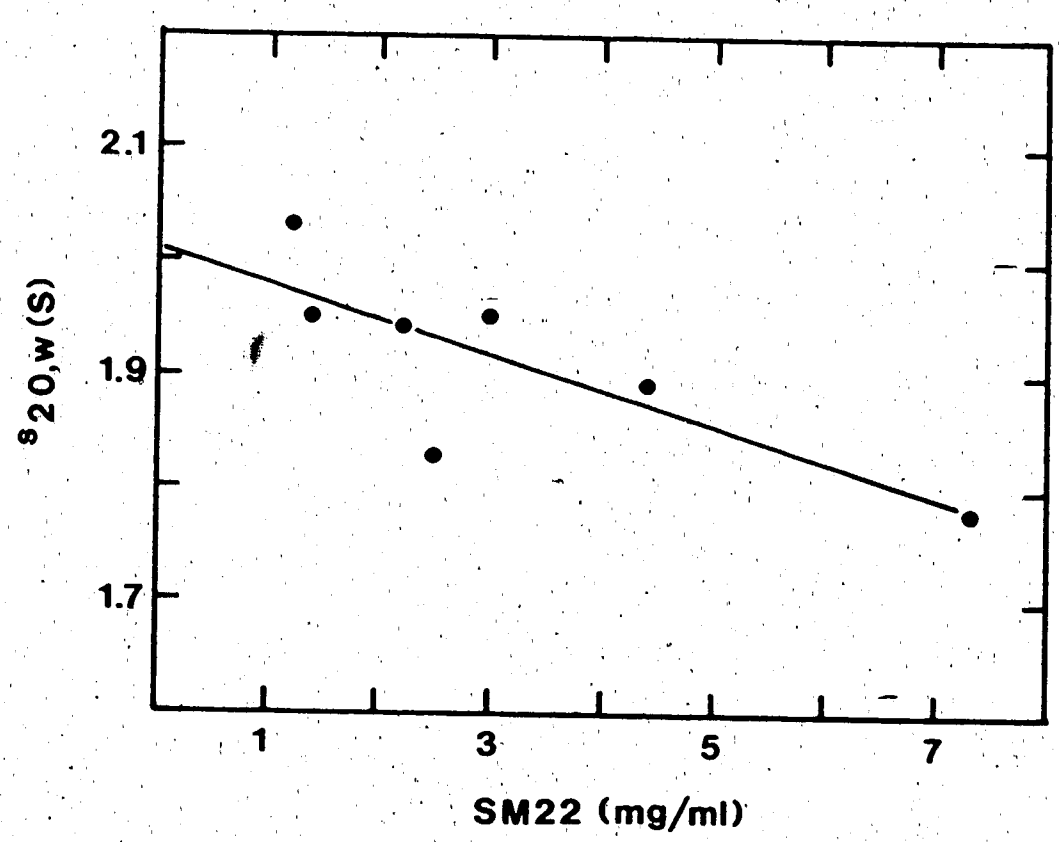


FIG. 29. Concentration dependence of the sedimentation coefficient of SM22. SM22 samples (0.4 ml) in KMED were sedimented at 60,000 rpm, using Kel F 2° centerpieces (12 mm) and schlieren photography.

sedimentation ($R_{s,sed}$) of 25.1 Å. A frictional ratio (f/f_{min} = 1.35) was determined for SM22α by dividing the average of $R_{s,gel}$ and $R_{s,sed}$ by the Stokes radius of an idealized spherical particle of identical M_r. When the estimated hydration of 0.38 g H_2O/g of protein is taken into account, the frictional ratio due to asymmetry alone (f/f_o) was found to be 1.18. Using a model for a prolate ellipsoid of revolution, this value gives an axial ratio of 4:1, indicating that the monomeric SM22α molecule is moderately asymmetrical.

The near-ultra violet CD spectrum of SM22α indicates that at least a portion of its aromatic residues are in a region of optical asymmetry (Fig. 30). The outstanding feature of this spectrum is the large positive band at 293 nm, which probably results from the 0-0 ^1L_b electronic transition of tryptophan which has been highly red-shifted from the normal position at 289.4 nm (Strickland, 1974). A smaller 0 + 850 cm^{-1} ^1L_b companion band is present at 284 nm. Three features of SM22's CD spectrum indicate that more than one tryptophan is involved in producing dichroism. These include the presence of fine structure from 295-310 nm, the intensity of the 293 nm band ($\Delta\epsilon$ = 3.6 $M^{-1}cm^{-1}$ where a maximum of 2.5 is expected for a single tryptophan) and the greater intensity of the 0-0 transition

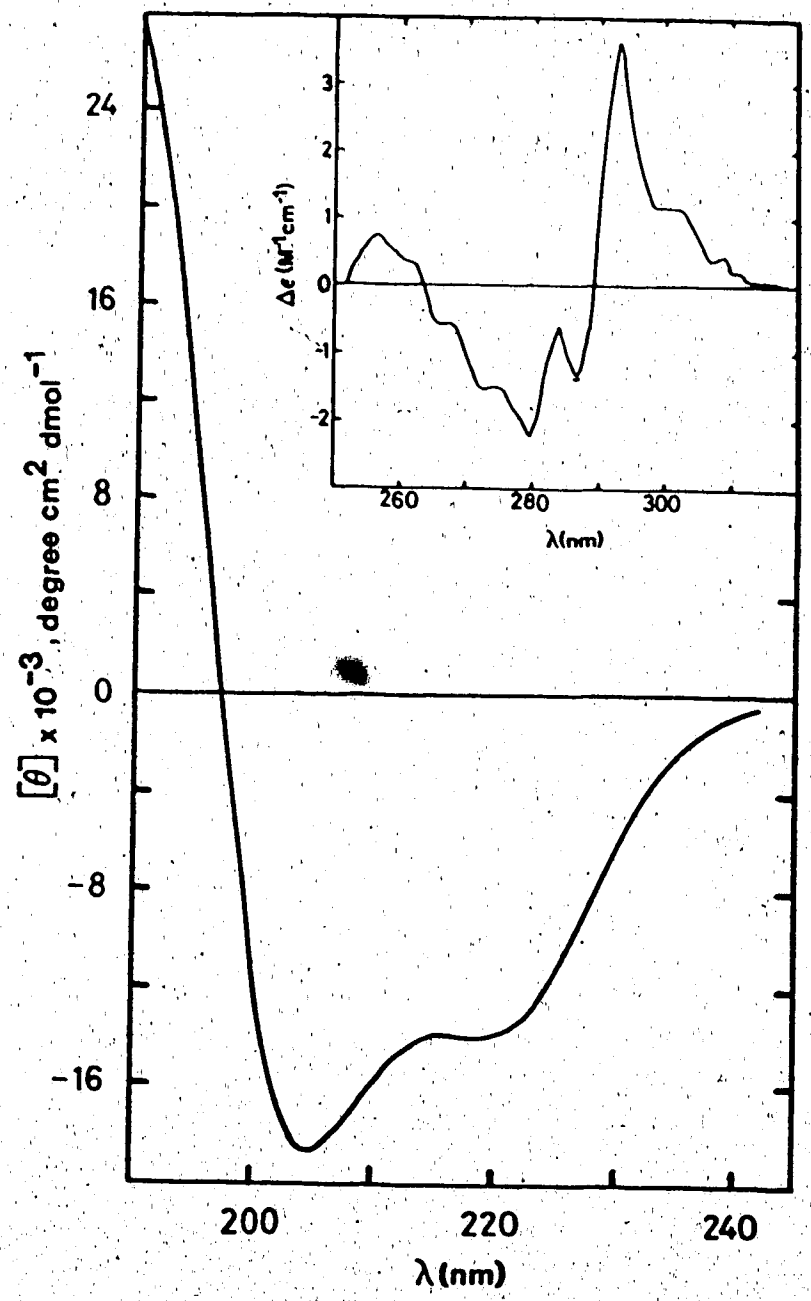


FIG. 30. Circular dichroic spectra of SM22α. Solutions for CD contained 0.54 mg/ml SM22α, 50 mM sodium phosphate (pH 7.0), 1.0 mM DTT. The path length was 0.0103 cm for the far ultraviolet and 1.0 cm for the near ultraviolet spectra. $\Delta\epsilon = \epsilon_L - \epsilon_R = [\theta]N/3300$ where ϵ_L and ϵ_R are the molar extinction coefficients for left and right circularly polarized light, [θ] is the mean residue ellipticity and N is the number of amino acid residues in SM22α (Strickland, 1974).

when compared to the 0 + 850 cm^{-1} transition (Strickland, 1974). The negative fine structure at 280 and 287 nm is likely due to tyrosine, as similar bands have been attributed to tyrosyl vibronic transitions in cytochrome c (Strickland, 1974). A shoulder at 275 nm may also result from tyrosine while structure at 256, 262, and 268 nm is probably due to phenylalanines. The far-UV CD spectrum of SM22α (Fig. 30) was analysed by computer comparison to proteins with known X-ray crystallographic structure (Provencher and Glöckner, 1981). The program indicated that SM22α contains 37% α-helix, 31% β sheet, 5% β turn and 27% random coil. The far-UV CD spectrum (215 nm-230 nm) of SM22 in a buffer containing 0.1 M KCl, 0.05 M sodium phosphate, pH 7.0, and 1 mM DTT was analysed for structural content by the method of Chen *et al.*, (1974) (not shown). It yielded 41 % α-helix, 34 % β-sheet and 25 % random coil.

Interaction Properties of SM22

In order to determine whether SM22 interacted with the contractile apparatus or resembled other proteins with similar physical properties, we carried out a series of binding assays (Table VII). Most of the assays were proven to be functioning properly by carrying out appropriate positive controls. As can be seen in Table VII, all the assays were negative.

TABLE VII Negative assays for SM22 function.

Substance assayed for binding to SM22	Technique	Positive control	Conditions
bromosulfophthalein (1 mg/ml)	gel filtration (a)	albumin	0.1 M KCl, 50 mM $NaPO_4$ pH = 7.0
ATP, ADP, AMP, GTP, GDP, GMP, cGMP, cAMP, CTP, CMP, CDP, NAD, NADH, FAD, NADP	^{31}P NMR (b)	none	25 mM Mops, pH = 7.2 150 mM KCl, 1 mM EDTA 1 mM EGTA, 2 mM DTT 0.7 mM SM22, 0.8 mM nucleotide + 0.1 - 5 mM $MgCl_2$
$^{45}Ca^{++}$	gel filtration (c)	Troponin C	50 mM Tris pH = 7.5 0.1 M KCl, 1 mM $MgCl_2$ 0.1 mM DTT, 13 µM $CaCl_2$
I^{125} Calmodulin	gel overlay (d)	Myosin Light chain Kinase	50 mM imidazole, pH = 7.0 0.15 M KCl, 1 mM DTT, (1 mM EGTA or 1 mM $CaCl_2$)
Troponin C - Sepharose	affinity chromatography (e)	C-terminal fragment of Troponin T (T2)	10 mM Tris, .01% NaN_3, .0.1 mM $CaCl_2$, 0.1 M NaCl
I^{125} Tropomyosin	gel overlay SM22 on the gel	Troponin T and Troponin I	50 mM imidazole, pH = 7.0 0.15 M KCl, 1 mM DTT
Whole gizzard extract	gel overlay with I^{125} SM22 α	I^{125} Calmodulin	"
actin	cosedimentation (f)	gizzard tropomyosin	100 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, 10 mM imidazole, pH = 7.8 actin 11.6 µM SM22 0.48 → 8.2 µM

Troponin C affinity chromatography was done by Joyce Pearlstone. Actin binding studies were done by Clive Sanders.

(a) Bernier and Jolles (1984)
(b) Nageswara Rao et al. (1978)
(c) Hummel and Dryer (1962)
(d) Carlin et al. (1981)
(e) Pearlstone and Smillie (1978)
(f) Eaton et al. (1975)
(T2) Residues 159-259 of troponin T

D. Discussion

In the comparison of smooth and skeletal muscles, important differences have been found in contractile properties, structure, metabolism and membrane physiology. One might expect that these differences would be manifest in the type and quantity of proteins present in the two tissues. Indeed, comparison of two-dimensional gels of smooth and skeletal muscle does indicate major alterations in protein composition (Hirai and Hirabayashi, 1983 ; Murakami and Uchida, 1985 and Hirabayashi, 1981). Of the proteins present in gizzard smooth muscle and absent from skeletal muscle, SM22 is the most abundant.

We have designed a simple scheme for both the purification of SM22 and the partial separation of its variants. This scheme can also be used for efficient purification of several other smooth muscle proteins. For example we have used column chromatography to purify histones from the distilled H_2O precipitate, myoglobin from the 70% $(NH_4)_2SO_4$ supernatant and albumin from the first peak of the CM-cellulose column profile (unpublished results). The initial extraction with 1 M KCl buffer at pH 7.0 was chosen to provide as complete extraction of SM22 as possible without recourse to denaturing conditions. As demonstrated in chapter 4 (Fig. 13 ; lane b), extraction of gizzards with a buffer containing 20 mM Tris, 1 mM $MgCl_2$, 1 mM DTT, pH 7.5, and 0.5 % Triton X -100 led to a significant but relatively low extraction of SM22. The recovery of SM22 in the extract

was increased significantly by including 40 mM NaCl in the same buffer (unpublished result). Significant levels of myosin, actin, myoglobin and tropomyosin were also recovered in these extracts. Further extraction of this residue with the same buffer but including 25 mM $MgCl_2$ and 60 mM NaCl led to close to quantitative extraction of the remaining SM22 along with SM21, myosin light chain kinase, caldesmon, filamin and some actin. CM-cellulose purification of SM21 from this extract has been shown to yield nearly pure SM22 as a by-product (Fig 15 A and 15 B ; fractions 100 to 120). A high $MgCl_2$ extraction could possibly be used as an alternative to the initial 1 M KCl extraction procedure described in this chapter.

Examination of selected fractions from the CM-cellulose chromatographic profile by one- and two-dimensional gel electrophoresis indicated the presence of at least three (α,β,γ) and perhaps four isoforms of SM22 (see Fig. 27). Of these α is the predominant and most basic form and constitutes about 70% of the total SM22 in this tissue with about 25% β. The β and γ components have slightly lower M_r as judged by their slightly faster migration rates on the SDS-polyacrylamide gels. However these do not seem to have arisen from proteolysis during the purification procedures since all three are present in a 9 M urea extract of the chicken gizzard tissue (see Fig. 25). A further variant (designated β') observed in Fig. 27 B and 27 C has the same apparent M_r as the α isoform but with the same charge

properties as the β isoform. Since this component was not resolved on two-dimensional gels of a 9 M urea extract of whole tissue (see Fig. 25) it may have arisen artifactually during the preparative procedures.

The possibility that these minor and more acidic isoforms may represent singly or multiply phosphorylated forms of the major α isoform was investigated by E. coli alkaline phosphatase digestion of both whole extracts of the tissue as well as fractions from the CM-cellulose chromatographic profile (see Fig. 27). No changes in the isoform distribution on two-dimensional gels were observed as a result of this treatment[2]

However SM22 can be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. While examining phosphorylation of myosin light chain kinase (in a high Mg^{2+} concentration extract of chicken gizzard) by this enzyme, Adachi *et al.* 1983 found that actin and a protein of lower M_r were also phosphorylated. We have subsequently observed that SM22 is the major lower M_r component of this extract and that purified SM22 is phosphorylated on a serine residue by the catalytic subunit of this enzyme.[3] However, the physiological significance of this observation is unclear since the level of phosphorylation obtained was never greater than 0.5 moles per mole of protein even after prolonged incubation at high enzyme to protein ratios.

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[2] Heeley, D.H. and Smillie, L.B. unpublished result.
[3] Heeley, D.H., Weber, M. and Smillie, L.B. unpublished data.

Examination of the phosphorylated SM22 on two-dimensional gels indicated that the singly phosphorylated species migrated in a position more acidic than the β isoform(Fig. 31), a further indication that the β isoform is not a phosphorylated derivative of α.

Amino acid analyses of the purified α and β isoforms also support the view that these are distinct entities (see Table V). While they are very similar, differences were observed in the half-cystine content (one in α and two in β) and in a higher level of basic amino acids in the α isoform. This latter difference probably accounts for the more acidic position of β on the two-dimensional gels and its earlier elution from the CM-cellulose chromatographic column. Failed attempts at automatic sequencing of the protein indicated a blocked NH_2-terminus.

The M_r of SM22, estimated at 22,000 by sedimentation equilibrium at close to physiological ionic strengths, is in reasonable agreement with the value of 22 to 24 kDa from SDS polyacrylamide gel electrophoresis. This agreement indicates that it is present as a monomer under these conditions. When the average of the Stokes radii (Rs) estimated from sedimentation velocity and gel filtration was used to calculate a translational frictional ratio (f/f_{min}) for SM22, a value of 1.35 was obtained. This may be compared with estimated values of 1.40, 1.53 and 2.4 for rabbit cardiac troponins-C, -I and -T respectively (Byers and Kay, 1982; Byers and Kay, 1983) and values of 1.2-1.3 typically observed for globular

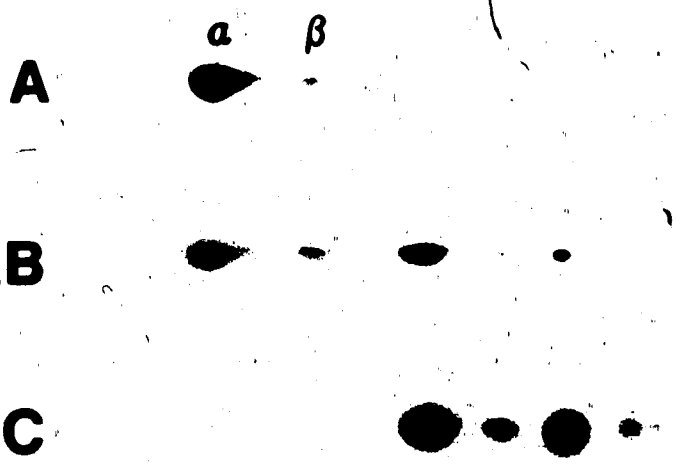


FIG. 31. Phosphorylation of SM22. (A) an NEPHGE gel, stained with coomassie blue, of SM22α slightly contaminated with SM22β. (B) As in A but after SM22 has been phosphorylated in a solution containing 0.05 mg/ml of the catalytic subunit of bovine cyclic-AMP dependent protein kinase, 2 mg/ml SM22, γ-^{32}P ATP (0.12 mM and 200 dpm/pmol), 20 mM Hepes, pH 7.5, 12 mM KCl and 3 mM $MgCl_2$ for 24 h at 30°C. (C) Autoradiograph of B (kindly provided by D.H. Heeley and L.B. Smillie.)

proteins (Edsall, 1953). This suggests that SM22 is a moderately asymmetric molecule. The estimates of 37% α-helix, 31% β sheet, 5% β turn and 27% random coil, derived from far ultraviolet CD measurements are also consistent with a globular structure. We have also examined the near ultraviolet CD spectrum of SM22. The spectrum produced by this technique depends on the structural environment of aromatic residues and thus is relatively unique for individual proteins. The near ultraviolet CD of SM22 is remarkably similar to that of azurin (Tang *et al.*, 1968), a protein that is involved in electron transport in *Pseudomonas aeruginosa* and whose tertiary structure is known from X-ray crystallographic studies (Adman *et al.*, 1978). These indicate that the near ultraviolet CD spectrum of azurin derives mainly from a single tryptophan residue in close contact with a phenylalanine and a single tyrosine, all within the hydrophobic confines of a β barrel structure. The major difference between the CD spectra of azurin and SM22 is the contribution of more than one tryptophan to the spectrum of SM22. It is therefore worthy of note that the CD spectrum of SM22 also closely resembles that of *Chironomus thummi* apohemoglobin 1 in which interaction between 2 tryptophans forms the basis of its major spectral characteristics (Strickland, 1974 and Wollmer and Buse, 1971).

Hirai and Hirabayashi (1983), using a specially constructed two-dimensional electrophoretic gel system (Hirabayashi, 1981) for the separation of chicken gizzard

smooth muscle proteins, observed an abundant basic 23 kDa protein of two isoelectric variants (isoelectric points of approximately 8.6 and 8.45). Developmental studies indicated that this protein, almost certainly identical to SM22, appeared by day 10 post fertilization, shortly after tropomyosin and desmin and at the same time as myosin light chain one. These observations indicate that SM22 is expressed in the developing chick embryo almost simultaneously with the other major proteins of the contractile apparatus. However, SM22 appears not to be related to any known protein with similar physical properties. Its amino acid composition is distinct from myokinase, troponin-I and a 23 kDa protein isolated from brain, all of which are monomeric basic proteins with similar M_r (Table V). We found that SM22 does not produce ATP from ADP, does not copurify with myokinase from smooth muscle (using an adaptation of the method of Scopes and Stoter (1982) for purification of myokinase from skeletal muscle), nor bind ATP, ADP, AMP or bromosulfophthalein, all properties shown by myokinase or brain 23 kDa.

The complete amino acid sequence of SM22 (Fig. 32) has been determined by Pearlstone *et al.* (1987). The protein consists of a single polypeptide chain of 197 residues, and has an M_r of 21,978 and net charge of +4.5 at neutral pH. A pattern of alternating hydrophobic and hydrophilic residues was found throughout the length of SM22, in agreement with its existence as a globular protein.

```
                    5                  10                  15
AcALA ASN LYS GLY PRO ALA TYR GLY MET SER ARG ASP VAL GLN SER

                   20                  25                  30
LYS ILE GLU LYS LYS TYR ASP ASP GLU LEU GLU ASP ARG LEU VAL

                   35                  40                  45
GLU TRP ILE VAL ALA GLN CYS GLY SER SER VAL GLY ARG PRO ASP

                   50                  55                  60
ARG GLY ARG LEU GLY PHE GLN VAL TRP LEU LYS ASN GLY ILE VAL

                   65                  70                  75
LEU SER GLN LEU VAL ASN SER LEU TYR PRO ASP GLY SER LYS PRO

                   80                  85                  90
VAL LYS ILE PRO ASP SER PRO PRO THR MET VAL PHE LYS GLN MET

                   95                 100                 105
GLU GLN ILE ALA GLN PHE LEU LYS ALA ALA GLU ASP TYR GLY VAL

                  110                 115                 120
VAL LYS THR ASP MET PHE GLN THR VAL ASP LEU PHE GLU ALA LYS

                  125                 130                 135
ASP MET ALA ALA VAL GLN ARG THR LEU VAL ALA LEU GLY SER LEU

                  140                 145                 150
ALA VAL THR LYS ASN ASP GLY HIS TYR HIS GLY ASP PRO ASN TRP

                  155                 160                 165
PHE MET LYS LYS ALA GLN GLU HIS LYS ARG GLU PHE SER GLU SER

                  170                 175                 180
GLN LEU LYS GLU GLY LYS ASN ILE ILE GLY LEU GLN MET GLY THR

                  185                 190                 195
ASN LYS GLY ALA SER GLN ALA GLY MET SER TYR GLY ARG PRO ARG

GLN ILE
```

FIG. 32. The amino acid sequence of SM22α from chicken gizzard smooth muscle. (From Pearlstone *et al.*, 1987).

The structural content of SM22 was predicted from its amino acid sequence using values based on conformational preference parameters. It was predicted to contain 31% α-helix, 24% β-sheet, 18% β-turn and 27% random coil, which agrees reasonably well with the predictions made from the CD spectrum. Pearlstone *et al.* (1987) used the FASTP program of Lipman and Pearson (1985) to compare the known amino acid sequence of SM22 to the National Biomedical Research Foundation's (Washington) protein sequence data base. Its sequence was not significantly similar to any previously determined protein sequence.

While no information is available on the cellular localization of SM22, the relative ease with which it is extracted by salt solutions indicates that it is not an integral membrane protein nor tightly associated with membranous structures. The presence of SM22 in extracts which contain elements of the contractile apparatus such as actin, tropomyosin, caldesmon, myosin light chain kinase, α-actinin and myosin (see Figs. 13 and 26) may indicate that SM22 is at least partially associated in the cell with one or more of these proteins. However in preliminary experiments we have been unable to demonstrate an interaction with F-actin, tropomyosin or calmodulin nor in fact, using radiolabelled SM22 in the gel overlay technique, with any protein component in a chicken gizzard muscle extract. Negative observations were also obtained for Ca^{2+} binding (gel filtration) and for interaction with a variety

of nucleotide derivatives (NMR). These apparent lack of interactions with individual contractile elements may indicate a requirement for multiple contacts in the normal *in vivo* activity of SM22.

While the functional properties of SM22 are presently obscure, its presence as a major component of smooth muscle tissues suggests an important role in their physiology. Its isolation and characterization in a pure form as described in this work should facilitate investigations directed towards an understanding of its interactive and biological properties.

VI. SM22 is Widely Distributed in Smooth Muscles. Purification from Bovine Aorta

A. Introduction

In the previous chapter we isolated and characterized an abundant basic 22 kDa protein (SM22) present in chicken gizzards at a molar ratio to actin of ≃1:3. While the cellular location and functional role of the protein is presently unknown, it is unique in its composition and properties and by amino acid sequencing shown to resemble no other previously characterized protein (Pearlstone *et al.*, 1987). To further assess the importance of SM22 as a smooth muscle component we have in this chapter demonstrated its presence in a variety of chicken smooth muscles by immunoblotting. Its presence in bovine aorta and porcine carotid has been shown by two-dimensional and SDS-polyacrylamide gel electrophoresis and its molar ratio to actin estimated to be 0.9:6.0 and 1.4:6.0 respectively. A procedure for purification of SM22 from bovine aorta is described and its amino acid composition shown to be similar to that of the chicken gizzard protein. We conclude that SM22 is widely distributed in smooth muscles as a major protein component.

B. Materials and Methods

Preparation of Antibodies and Immunoblotting

The α isoform of chicken gizzard SM22 was prepared as previously described in chapter 5. For the raising of polyclonal antibodies, 200 μg of SM22α dissolved in 500 μl of water and 500 μl of Freund's complete adjuvant was injected into a rabbit, half subcutaneously and half intramuscularly. Further injections of 200 μg in Freund's incomplete adjuvant were at 10 day intervals for 3 months. Production of antibodies was monitored by enzyme linked immunoabsorbent assay (Engvall, 1980), followed by their isolation on a protein A affinity column. Immunoblotting was by the method of Towbin *et al.* (1979). SDS-urea extracts of chicken tissues were electrophoresed on SDS-polyacrylamide gels(13 %), using the buffer system of Laemmli (1970). Following electrophoretic transfer to nitrocellulose (Schleicher and Schuell) and incubation with anti-SM22α antibody at a dilution of 1:5000, the reactive proteins were detected with goat antirabbit IgG-alkaline phosphatase (Sigma) and a solution containing 1 mg/ml fast red TR salt (Sigma), 1 mg/ml naphthol AS-B1 phosphate (Sigma), 0.1 M Tris, pH 9.2 and 5 mM $MgCl_2$ (O'Connor & Ashman, 1982).

Electrophoresis and Gel Scanning

Bovine aortas and porcine carotids were kindly donated by Gainers Inc. (Edmonton). SDS-urea extracts were run on

SDS-polyacrylamide gels (13 %) and the relative amounts of actin, SM22 and tropomyosin determined using a Joyce Loebl Chromoscan 3 scanning densitometer as described in General Materials and Methods. Relative staining intensities with coomassie blue were based on standard curves for rabbit skeletal actin and chicken gizzard tropomyosin and SM22. Identification of aorta proteins on the gels was made according to their relative abundance and migration properties as described for actin, myosin and tropomyosin (Cohen & Murphy, 1978), vimentin and desmin (Gabbiani *et al.*, 1981) and caldesmon (Clark *et al.*, 1986). Filamin was tentatively identified by comigration with its gizzard counterpart and albumin was purified from the 70% saturated $(NH_4)_2SO_4$ supernatant described below and identified from its amino acid composition. In all cases the protein identities were checked by two-dimensional gel electrophoresis and/or purification properties. Two-dimensional gels were run with non-equilibrium pH gradient electrophoresis in the first dimension as described by O'Farrell *et al.* (1977).

Purification of SM22 from Bovine aorta

Chilled aortas were dissected of external fat and connective tissue and minced in a grinder with 2.5 mm holes. The initial stages of purification were as described for chicken gizzards (chapter 5) up to and including CM-cellulose chromatography at pH 4.7 (see Fig. 38). Since

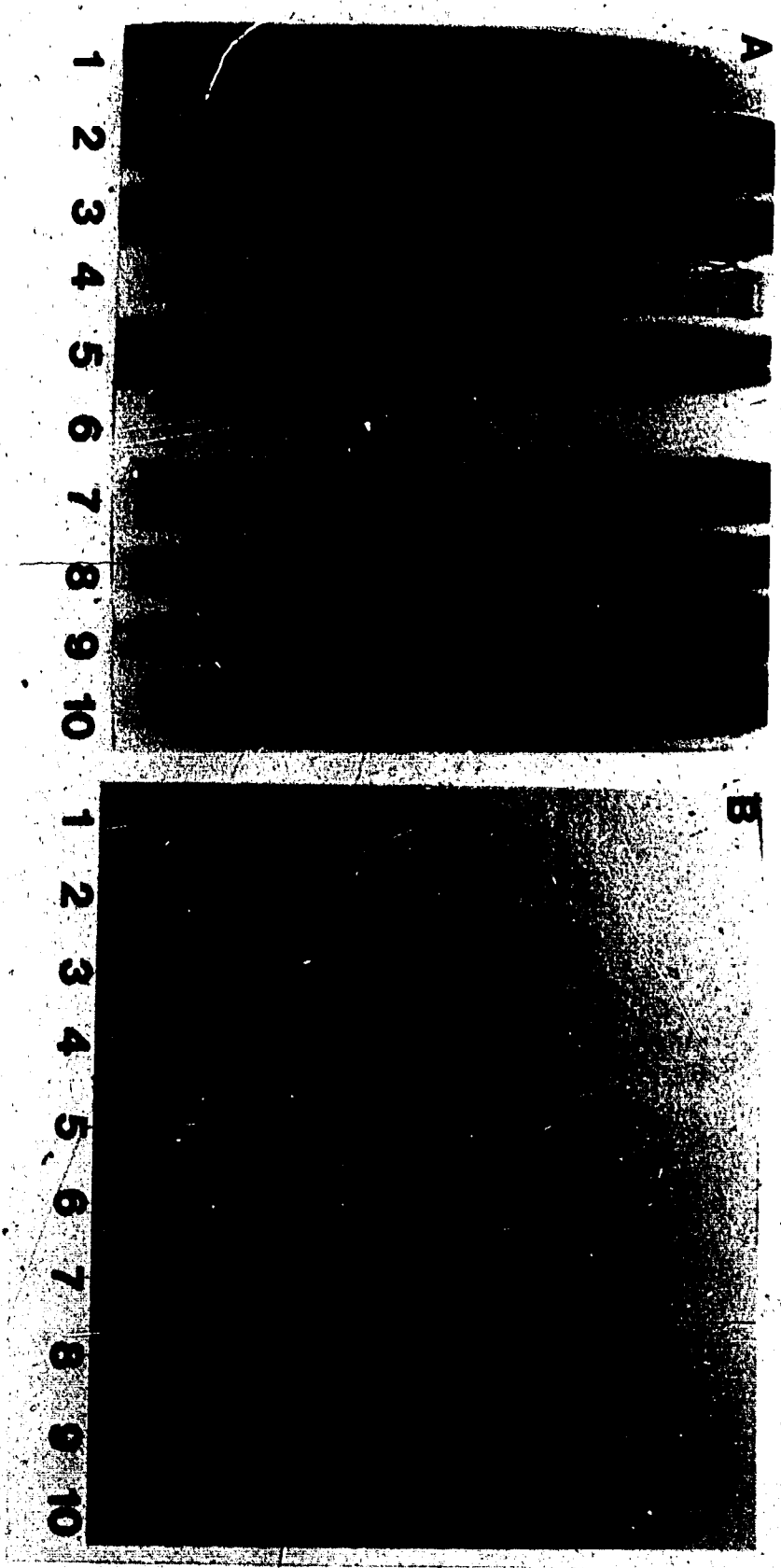
the aorta protein was not homogeneous at this stage, a further purification step was carried out on a 2.5 x 20 cm column of Affi-gel Blue (Biorad) at pH 8.0 (see Fig. 39).

C. Results

Detection of SM22 in SDS-urea Extracts of Various Organs

We have used the immunoblotting procedure of Towbin *et al.*, (1979) to investigate the presence or absence of SM22 in SDS-urea extracts of a variety of organs from the chicken and of bovine aorta. Rabbit anti-SM22 antibodies were found to bind to a 22 kDa protein in extracts of chicken organs that contain smooth muscle (Fig. 33). These included intestine, uterus, gizzard, esophagus, and aorta. With overloading (not shown) trace amounts of SM22 were observed in heart, brain and liver but none was detected in breast muscle. Anti-SM22 antibodies also cross reacted with a 70 kDa protein in esophagus which we have been unable to identify. The antibodies to gizzard SM22 did not cross react with an SDS-urea extract of bovine aorta or with purified SM22 from this source (Fig. 34). A very weak cross reaction, which appeared to occur with the heavily overloaded actin band, was seen in the whole extracts in Fig. 34. This anomaly was not seen with the actin standard in Fig. 34, lane 4 or in Fig. 33.

Fig. 33. Immunoblot of chicken organ extracts. (A) 10-18% SDS-polyacrylamide gel of chicken organ SDS-urea extracts, stained with Coomassie Blue; intestine (1), uterus (2), heart (3), gizzard (4), liver (5), SM22 (6), brain (7), esophagus (8), skeletal muscle (9), aorta (10). (B) Nitrocellulose transfer of a gel with the same composition as in A and immunostained for SM22 as described in Materials and Methods. Sample loadings are approximately 50 μg per lane with the exception of SM22 (0.5 μg), gizzard (15 μg), and skeletal muscle (100 μg).


A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9 10

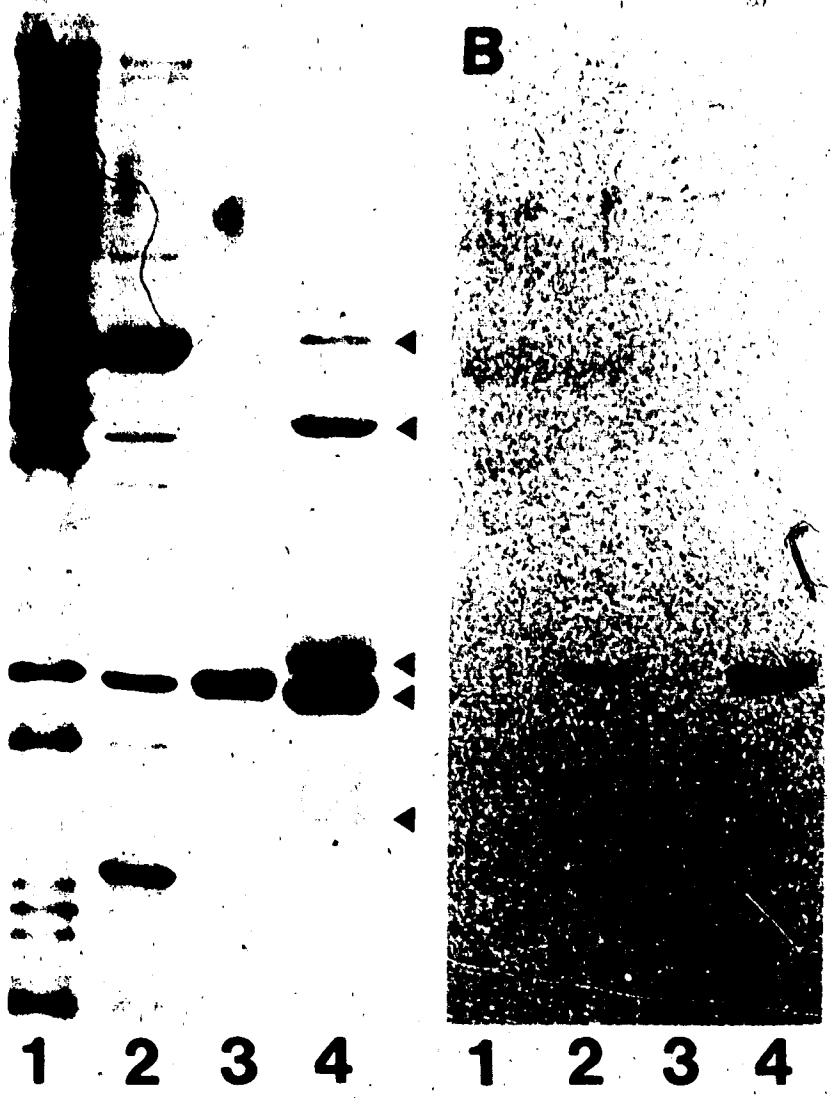


FIG. 34. Immunoblot of an SDS-urea extract from bovine aorta. SDS-urea extracts of bovine aorta, 40 μg (1) and chicken gizzard, 20 μg (2) were electrophoresed on 13 % polyacrylamide gels and transferred to nitrocellulose. Other lanes include, aorta SM22, 1 μg (3) and standards (arrowheads) from top to bottom:actin (42,000), rabbit skeletal troponin T (38,000), troponin I (24,000), gizzard SM22, and troponin C, (17,000) (4). (A) stained with amido black; (B) immunostained for SM22.

Presence of SM22 in Mammalian Arteries

SDS-urea extracts of bovine aorta and porcine carotid arteries exhibited a major 22 kDa band (SM22) when electrophoresed on SDS-polyacrylamide gels (Fig. 35). The molar ratio of actin:SM22:tropomyosin is 6.0:0.9:1.0 in bovine aorta and 6.0:1.4:1.0 in porcine carotid. This may be compared with ratios of 6.5:2.0:1.0 in chicken gizzard (chapter 5). SM22 was also detected in non-equilibrium, two-dimensional gels of urea extracts from mammalian arteries (Fig. 36). When the most basic variant, (α), of gizzard SM22 was co-electrophoresed with a 9 M urea extract of pig carotid it was indistinguishable, in both apparent M_r and pI from the most basic variant of SM22 in the carotid tissue. The pig carotid SM22, like the gizzard SM22, appears to consist of at least three variants, designated α, β and γ in order of decreasing basicity. The relative level of the β and γ components in this tissue is higher (ratio of α:β:γ $\simeq$2.5:2:1) than in the chicken gizzard (ratio of 14:5:1) (chapter 5). In the case of bovine aorta, the α:β:γ ratio was also $\simeq$2.5:2:1 and a still more acidic variant, δ (Fig. 36 A, inset) was detected when higher loadings of the extract were applied to the two-dimensional gel. This variant was not seen in the chicken gizzard extracts.

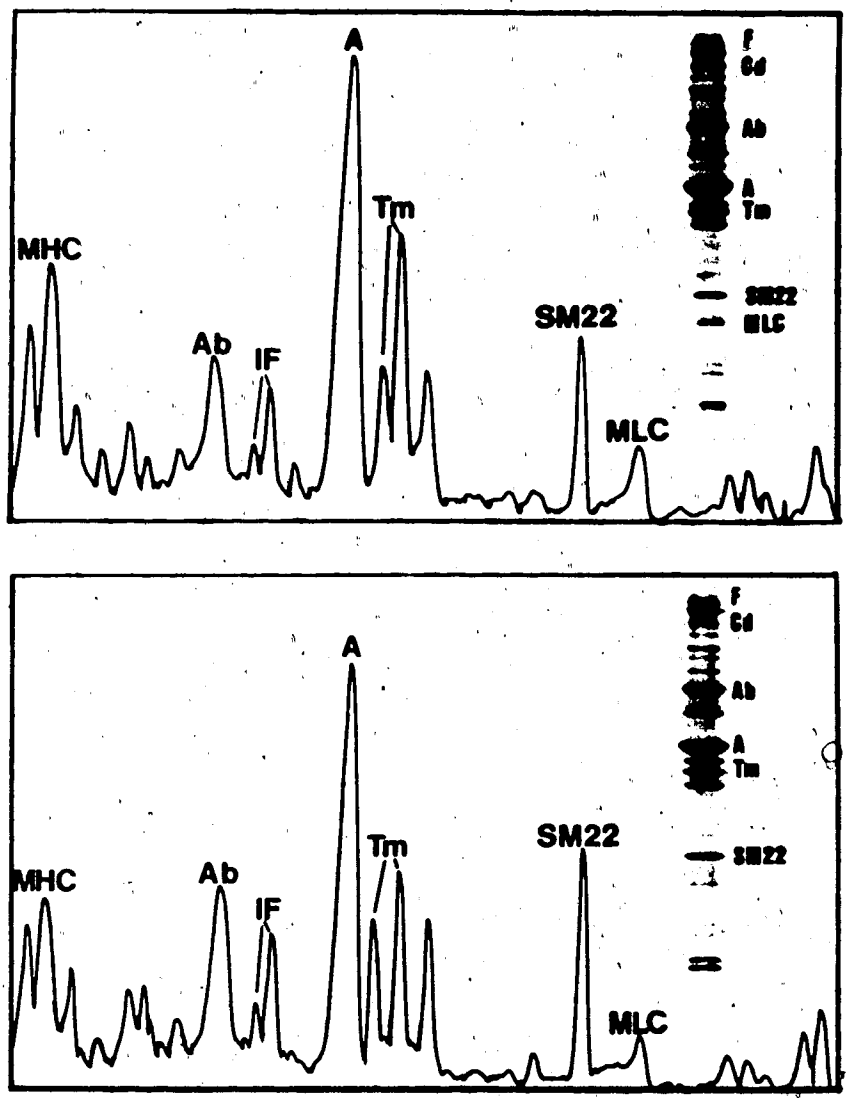


FIG. 35. Densitometric scans of vascular tissue extracts. SDS-urea extracts of bovine aorta (A) and porcine carotid (B) were run on 13% SDS-polyacrylamide gels. Filamin (F), myosin heavy chain (MHC), caldesmon (Cd), albumin (Ab), intermediate filament proteins vimentin and desmin (IF), actin (A), tropomyosin (TM), myosin light chain (MLC). The insets are photographs of the gels that were scanned.

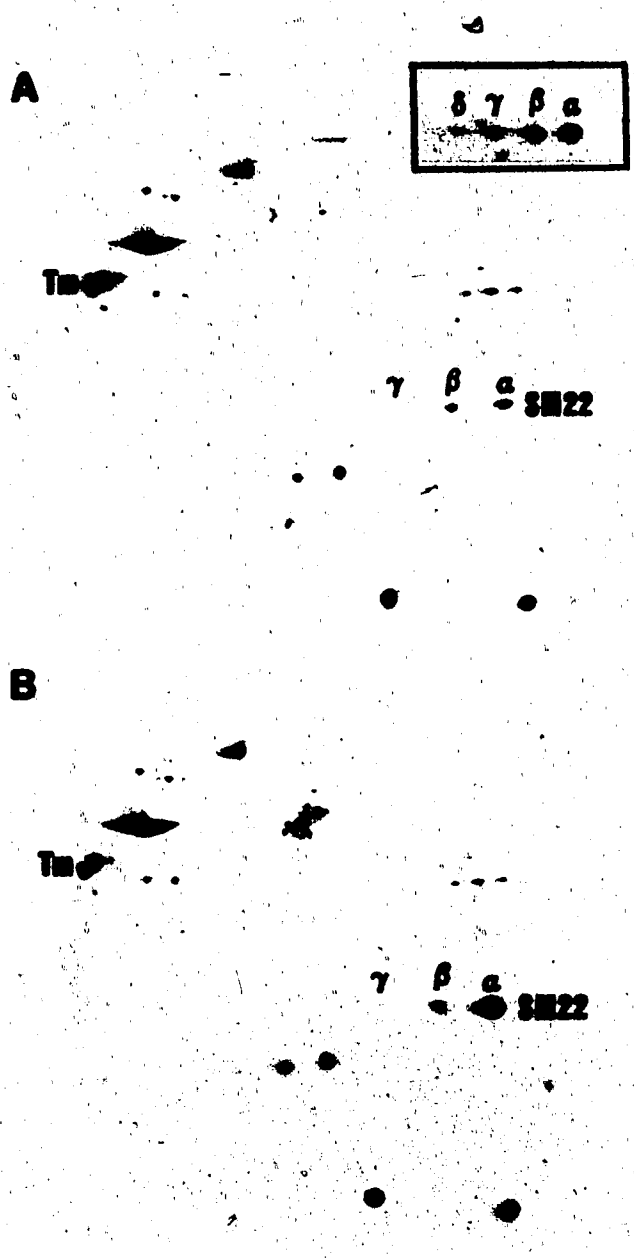


FIG 36. Two-dimensional gels of a 9 M urea extract of porcine carotid (A) and porcine carotid with added chicken gizzard SM22 α (B). The horizontal dimension is non equilibrium isoelectric focussing carried out for 3000 Vh in the presence of pH 3-10 ampholines (LKB). The vertical dimension is 14% SDS-polyacrylamide gel electrophoresis. The inset is SM22 cut out from a two-dimensional gel of bovine aorta. α, β, γ and δ indicate the variants of SM22 from most basic to most acidic.

To provide further evidence for the identity of SM22 in bovine aorta we have purified a mixture of its α and β isoforms through an extension of the procedure used for its purification from chicken gizzards. This procedure was essentially the same as that previously described in chapter 5, up to and including precipitation with 70% saturated $(NH_4)_2SO_4$ (Fig. 37). At this stage the 22 kDa band was heavily contaminated with several other proteins made up primarily of a 58 kDa component and a basic 20 kDa protein (SM20). Subsequent CM-cellulose chromatography (Fig. 38) achieves a considerable increase in purity of SM22, but, unlike the case with the gizzard extracts, leaves it still contaminated with the 58 and 20 kDa proteins. An additional purification step on Affi-blue gel (Fig. 39) however was successful in yielding an SM22 preparation that was >90% pure, comigrated with chicken gizzard SM22 on SDS polyacrylamide gels (Fig. 34) and consisted primarily of a mixture of α and β isoforms (not shown). Amino acid analysis of this product (Table VIII) indicated that its composition was very similar to that of the chicken gizzard SM22 α and β isoforms.

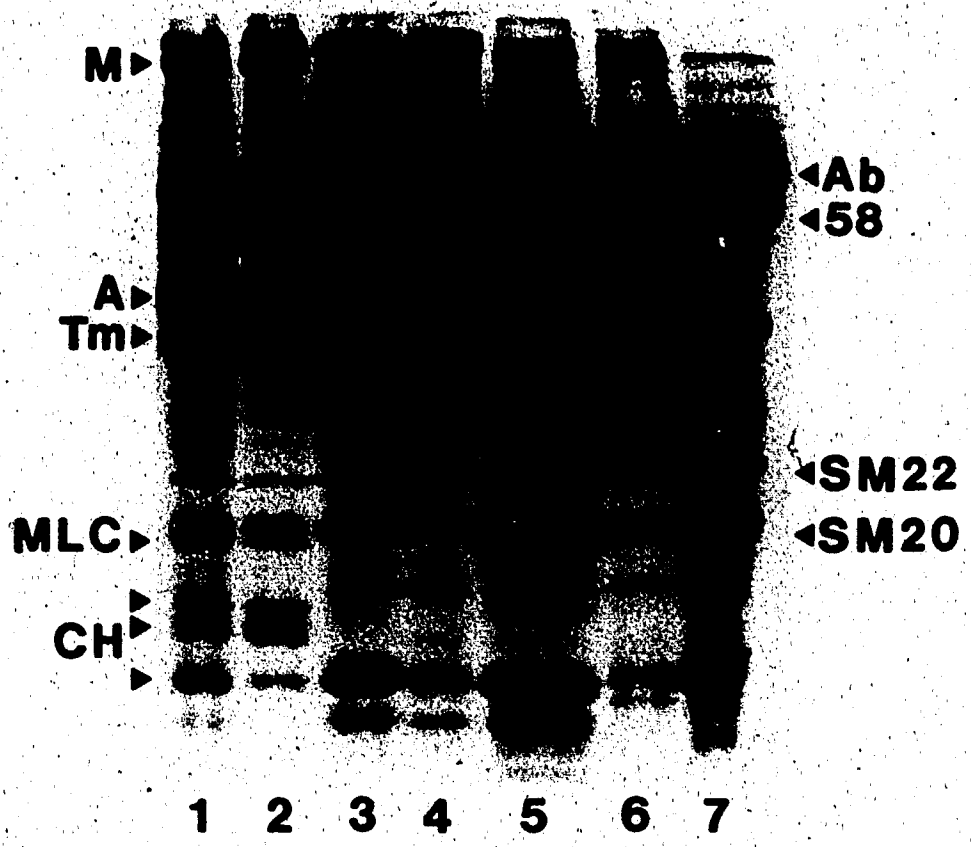


FIG. 37. SDS-PAGE of fractions at various stages of aorta SM22 purification. Lanes are (1) 1 M KCl extract of bovine aorta, (2)distilled H_2O precipitate, (3) distilled H_2O supernatant, (4) pH 4.7 supernatant, (5) pH 4.7 precipitate, (6) 35 % $(NH_4)_2SO_4$ precipitate, (7) 70 % $(NH_4)_2SO_4$ precipitate. Abbreviations are as in Fig. 3 with the addition of (CH) core histones from top to bottom H3, H2A and H2B, and H4 ; (58) unidentified 58 kDa band.

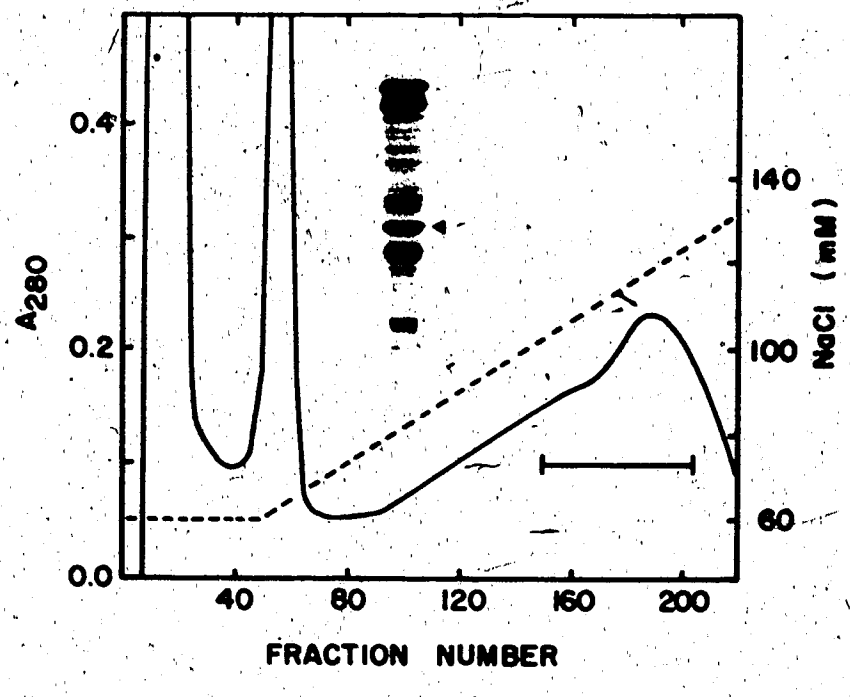


FIG. 38. CM-cellulose chromatographic purification of bovine aorta SM22. The 70% saturated $(NH_4)_2SO_4$ precipitate was taken up in 120 ml of buffer (60 mM NaCl, 50 mM sodium acetate, 1 mM EGTA, pH 4.7), dialysed against the same buffer and applied to a 5 x 45 cm column, previously equilibrated with the same buffer. The column was then developed with 1 liter of the same buffer followed by a 3 liter linear NaCl gradient (60 to 135 mM). Flow rate, 120 ml/h; fraction size, 18 ml. Inset shows SDS-polyacrylamide gel of the 70% saturated $(NH_4)_2SO_4$ fraction applied to the column. Fractions were pooled as indicated.

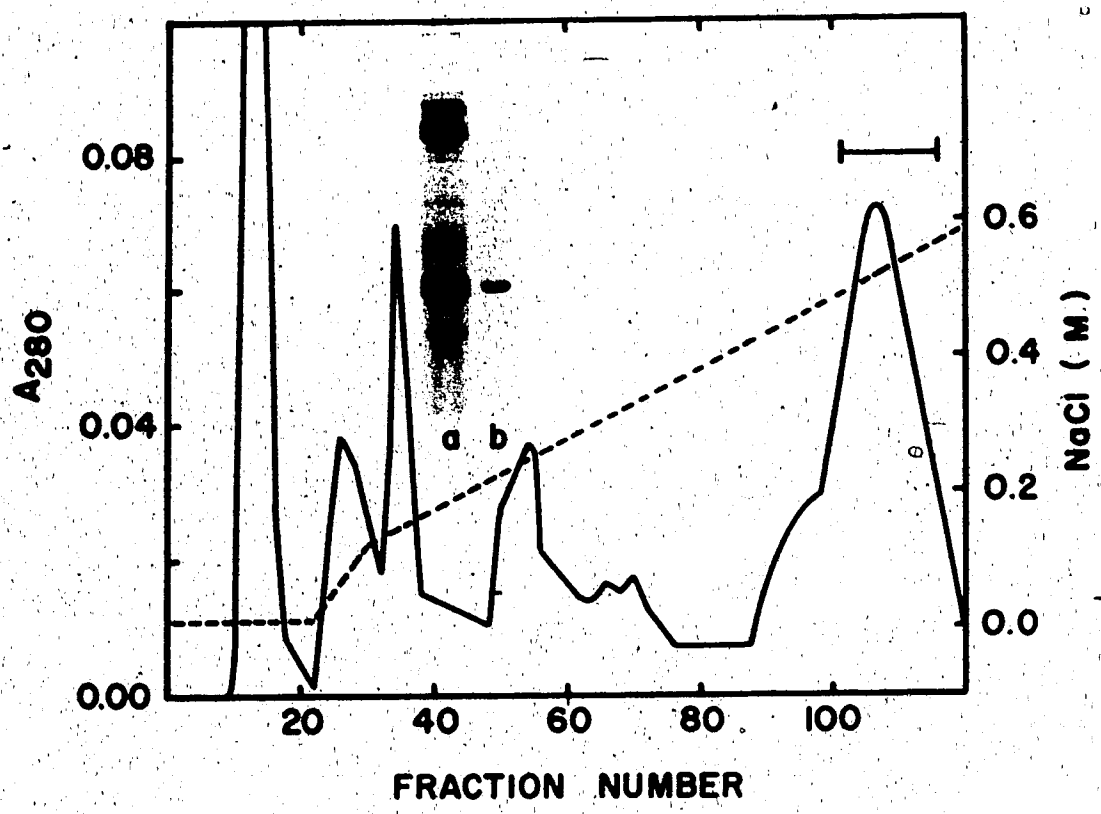


FIG. 39. Affi-gel blue chromatographic purification of SM22. One hundred mg of the dialysed and lyophilized material pooled in Fig. 38 was dissolved in 30 ml of buffer (20 mM N-2-hydroxyethylpiperazine, N^1-2 ethanesulfonic acid (Hepes), pH 8.0, 10 mM NaCl, 1 mM EDTA, 1 mM DTT) and applied to a 2.5 x20 cm column, previously equilibrated with the same buffer. The column was then developed with 250 ml of the same buffer followed by a 1 liter linear NaCl gradient (0.1 to 0.6 M). Flow rate, 40 ml/h; fraction size, 10 ml. Inset gel lanes show material applied to column (a) and that recovered from pooled fractions (b).

TABLE VIII

Amino acid composition of SM22 from bovine aorta

The composition of aorta SM22, which was approximately a 60:40 mix of α and β variants, was based on 11 alanines in order to approach the sedimentation equilibrium Mr of gizzard SM22α (22,000). Samples of SM22 were hydrolysed for 24, 72 and 120 hrs. Values for serine and threonine were extrapolated to 0 time. Valine and isoleucine were taken from the average of 72 and 120 hr hydrolysates. Values for SM20 were calculated from its apparent Mr of 20,000.

Residue	Aorta SM22	Gizzard SM22α[a]	Gizzard SM22β[a]	Aorta SM20
Asx	16	19	19	16
Thr	6.9	6.2	6.8	6.9
Ser	12	13	13	9.3
Glx	26	25	24	25
Pro	9.5	9.5	9.6	8.9
Gly	20	18	17	15
Ala	11	14	14	10
Val	16	14	14	15
Met	7.7	7.5	6.8	6.7
Ile	5.0	6.5	6.5	4.8
Leu	15	14	14	14
Tyr	5.7	6.0	5.8	4.5
Phe	6.0	7.1	6.9	6.9
His	3.2	3.2	3.3	3.5
Lys	16	18	17	18
Arg	11	9.2	8.4	8.4
Trp	n.d.[b]	3.0	2.9	n.d.
Half-Cys	n.d.	1.1	2.2	n.d.

[a] See Chapter 5

[b] n.d.; not determined

Purification of Aorta SM20

We have previously noted the presence of a basic 20 kDa protein in chicken gizzards (Fig. 11). By two-dimensional electrophoresis this protein appears to be more abundant (relative to SM22) in aorta than in gizzard (compare Fig. 11 with Fig. 36). In order to purify SM20 from bovine aorta, we pooled fractions 61 to 71 from CM-cellulose chromatography (Fig. 38). This material was chromatographed on Affi-Gel Blue and SM20 was obtained in ≈90 % purity (Fig. 40 ; pool II). Its amino acid composition was similar to that of SM22 (Table VIII). An attempt to determine its NH_2-terminal amino acid sequence was unsuccessful, indicating that it was blocked as is that of SM22. We have also noted that SM20 increases in abundance relative to SM22 in frozen gizzards compared to fresh (unpublished observation). It is possible that SM20 results from endoproteolytic cleavage of SM22 in its COOH-terminal region to produce a truncated version of the latter protein.

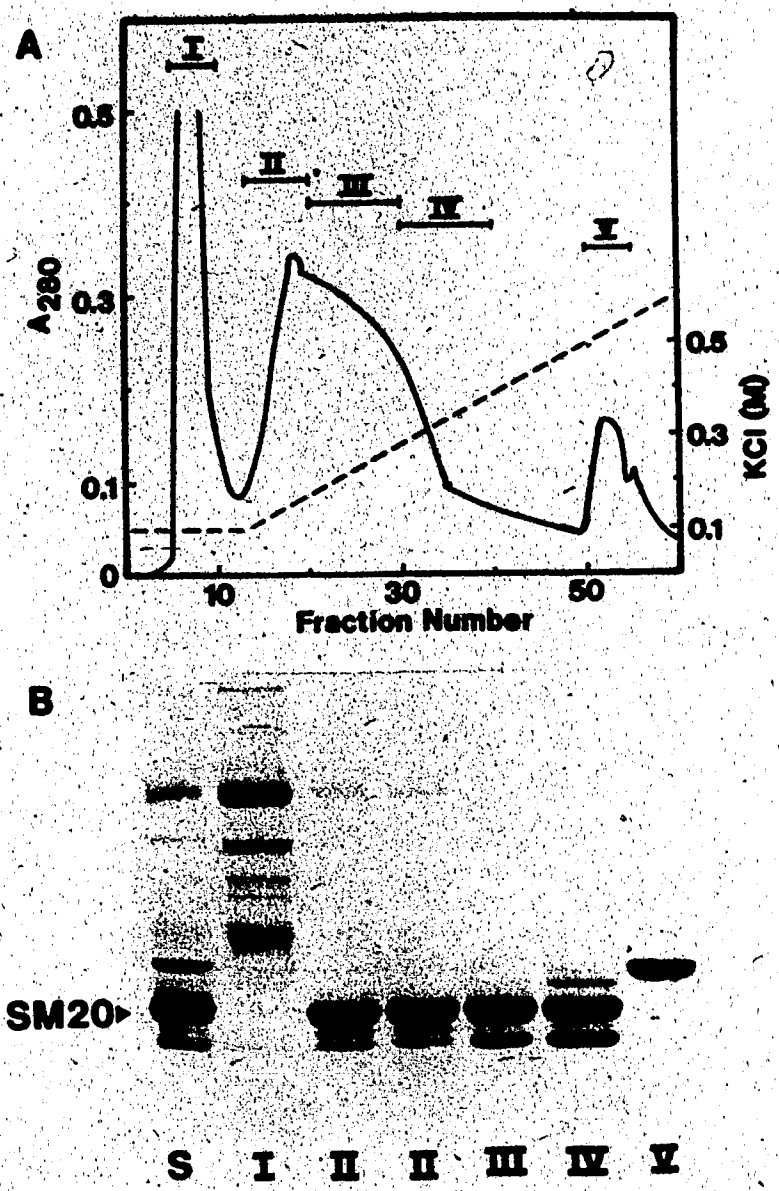


FIG. 40. Affi-gel blue chromatographic purification of aorta SM20. (A) Chromatographic profile. Fifty mg of protein pooled from fractions 61 to 71 of Fig. 38 was dialysed against 2 l of buffer containing 20 mM Hepes, pH 8.0, 0.1 M NaCl, 1mM EDTA and 1mM DTT, and applied to a 1.2 by 10 cm column, previously equilibrated with the same buffer. The column was then developed with 20 ml of the same buffer followed by a 100-ml linear NaCl gradient (0.1 to 0.6 M). Flow rate 20 ml/h ; fraction size 2 ml. Fractions were pooled into 5 groups I to V. (B) SDS-PAGE of pooled fractions from A. (S) starting material.

D. Discussion

Many of the abundant smooth muscle proteins have now been purified and characterized. While most of this work was done initially with chicken gizzards the recent purification and characterization of proteins such as myosin light chain kinase (Hathaway *et al.*, 1985), caldesmon (Clark *et al.*, 1986), actin (Strzelecka-Golaszewska *et al.*, 1985) and myosin (Chako & Rosenfeld, 1982) from mammalian vascular smooth muscle is indicative of the growing interest in this physiologically important tissue. Indeed, the first observation that myosin light chain phosphorylation does not correlate with maintenance of tension (Driska *et al.*, 1981; Barany *et al.*, 1985) in smooth muscle was made with strips of vascular tissue. These findings indicated the requirement for a second control mechanism in smooth muscle. Some possibilities that have been proposed but not proven include binding of Ca^{2+} to myosin (Chako & Rosenfeld, 1982), thin filament linked control by leiotonin (Ebashi, 1980) and a calmodulin-linked Ca^{2+} regulation by caldesmon (Marston & Smith, 1985).

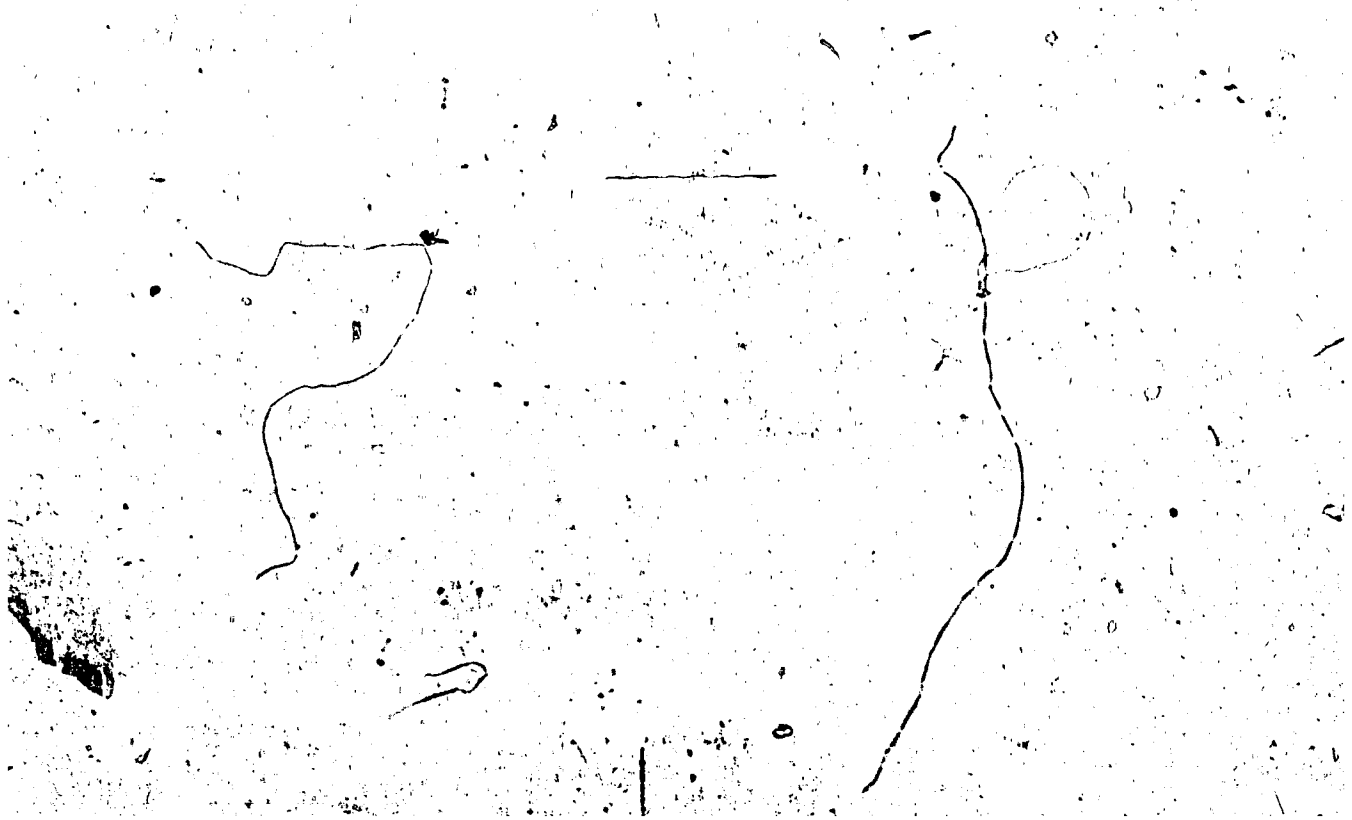
Surprisingly, little attention has been paid to the possibility that low M_r (below 30,000) proteins other than the myosin light chains and calmodulin may play a role in the control of smooth muscle contraction. However, a recent study (Rapoport *et al.*, 1982) has demonstrated the phosphorylation of several low M_r components in intact vascular smooth muscle, other than the myosin light chain.

The possibility that these or other low M_r protein components may contribute to the regulation of smooth muscle metabolism deserves further investigation.

In chapter 5 we purified and characterized SM22 from chicken gizzard. This protein has an apparent M_r on SDS-polyacrylamide gels of 22,000 and is distinct from any previously characterized protein. In the present study we have made polyclonal antibodies to gizzard SM22 and used them in the immunoblot procedure to test for the presence of SM22 in extracts from a variety of chicken organs. The results show that SM22 is present primarily in organs that contain abundant smooth muscle tissue (Fig. 33) and are therefor consistent with SM22 being a smooth muscle specific protein. Immunohistochemical studies will however be required to substantiate this observation. We found that our rabbit anti chicken SM22 antibodies did not cross react with extracts from bovine aorta (Fig. 34). One can however detect an abundant protein on two dimensional gels of bovine aorta and porcine carotid extracts with the same M_r and isoelectric point as SM22 from chicken gizzard (Fig. 36). We have purified SM22 from bovine aorta using a procedure almost identical to that used for gizzard SM22 (Fig. 37). However the presence of major contaminants after CM-cellulose chromatography in the aorta preparation (Fig. 39 ; inset) made necessary a further purification step by Affi-gel blue column chromatography.

Amino acid analysis of a mixture of α and β isoforms of aorta SM22 (Table VIII) indicate that this protein is similar to gizzard SM22 despite its lack of antibody cross-reactivity. The isoform distribution of vascular and gizzard SM22 is also similar, although the more acidic β and γ subunits are relatively more abundant in vascular tissue (α:β:γ; 2.5:2.0:1.0). A fourth still more acidic variant (δ), not detected in chicken gizzard, is present in aorta. The relative molar abundance of SM22 compared to actin and tropomyosin in vascular tissue is about 1/2 that found in gizzard.

We conclude that SM22 is present as a major component in the smooth muscles of both birds and mammals. Although the function of SM22 remains unknown its abundance suggests an important role in the physiology of smooth muscle tissue.



VII. Summary and Future Prospects

Smooth muscle is an important component of many vertebrate internal organs. It has understandably been subjected to intense pharmacological characterization. The biochemical characterization of smooth muscle was however only begun in earnest during the 1970s. Much remains to be learned about metabolism, contractile activity, and excitation-contraction coupling in this tissue. In order to understand these processes we will have to identify and characterize the proteins that carry them out.

In the vast majority of cases, proteins are purified because they have been associated with a specific function. However with modern techniques of gel electrophoresis and recombinant-DNA technology, we can identify many proteins without knowledge of their function. It can, unfortunately, be extremely difficult to determine the function of these proteins. Some approaches to this problem will be discussed below.

We began our study of smooth muscle by searching for tropomyosin binding proteins, with a special interest in identifying troponin-like components. ^{125}I-tropomyosin gel overlay studies led to the discovery of SM21. After initial difficulties in characterizing SM21 following a denaturing purification, we developed a non-denaturing purification protocol. SM21 is a moderately asymmetrical globular monomer that is strongly basic and possesses a distinctive amino acid composition (high in half-Cys and Gly) and NH_2-terminal

amino acid sequence. It is present in medium abundance in both chicken gizzard (≃ 1 : 16 with actin) and bovine aorta smooth muscles. Although the gel overlay indicates that SM21 can bind to tropomyosin, actin and calmodulin, its amino acid composition and NH_2-terminal sequence do not resemble that of troponin I which also binds to all of these proteins.

In addition to binding to actin, calmodulin and tropomyosin, SM21 also co-purifies with several contractile apparatus associated proteins such as MLCK, caldesmon and filamin. These findings indicate a particular need for two types of further research.

(1) Actomyosin ATPase studies should be carried out, preferably with smooth muscle components. These studies should be carried out with both dephosphorylated, and phosphorylated myosin, and in the presence and absence of other potential smooth muscle regulatory proteins, such as caldesmon and leiotonin.

Immunofluorescence studies should be used to determine the location of SM21 in cells. If SM21 can be associated with a distinct organelle or filament system, then progress towards determining its actual function will be greatly facilitated.

While purifying SM21 we inevitably ended up with fairly pure SM22. One of the reasons for this finding is that SM22 is extremely abundant (≃ 1 : 3.3 molar ratio to actin). After increasing the yield and purity of the SM22

preparation, we embarked on the task of finding its function. One possibility was that structure would give us a clue.

SM22 is a moderately asymmetrical globular protein. Its amino acid composition is not distinctive but its near-UV CD spectrum does show interesting similarities with two metal binding proteins, azurin and *Chironomous* hemoglobin. The sequence of SM22 was not similar to any previously determined protein sequence (Pearlstone *et al.*, 1987).

SM22 is interesting in the absolute negativity it displays in attempts to define its function. At concentrations near mM, it had no detectable effect on a wide range of nucleotide phosphate resonances (NMR). In a gel overlay study, ^{125}I-SM22 showed no binding to any protein in an SDS-urea extract of chicken gizzard. It is possible that the most acidic isoforms of SM22 (γ and δ) might result from phosphorylation of the more basic isoforms. However we were unable to alter the isoform composition of SM22 with alkaline phosphatase or demonstrate that SM22 is phosphorylated *in vivo*. SM22 is widely distributed amongst smooth muscle containing organs in chicken and is also found in bovine aorta and pig carotid. We believe that it plays an important role in smooth muscle. In order to discover this role it will be useful to carry out actomyosin ATPase assays and immunohistochemistry as mentioned previously for SM21. Other studies should include:

(1)X-ray crystallography ; determination of the structure of SM22 may reveal a structural element that is similar to that of a protein of known function.

(2) CIoning ; elucidation of the transcript and gene structure of SM22 might reveal the existence of non-transcribed sequences that are shared with proteins of known function. In addition it would help to resolve the origin of the SM22 isoforms. In the case of SM21, cloning would also be useful for obtaining the remainder of the sequence.

(3)Both SM21 and SM22 should be tested for the ability to effect tension production and maintenance in skinned smooth muscle fibers. It would be especially interesting to determine their effect on the maintenance of tension in the presence of CTP (Hoar *et al.*, 1985).

The problem with all of the above studies is that they may turn out to be negative. In the case of X-ray crystallography and cloning a negative result will not be disastrous as the information gained will still be useful when the function is found. With ATPase and tension assays I would suggest that the work be carried out in conjunction with another project.

In summary, we have purified and characterized two novel proteins from smooth muscle. While we have not been able to clearly define their function, we have provided considerable data which should help in the completion of this task. In the course of this work we have helped to

clarify the composition of the high mobility proteins of smooth muscle and to provide information that should be helpful in purifying some of the uncharacterized components. We have also provided further evidence against the existence of proteins in smooth muscle that resemble troponin I or T by the criteria of M_r, primary sequence and abundance. SM32 is probably the only remaining candidate that might possibly meet all of these criteria.

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