The University of Manitoba

PROTEOLYTIC STUDIES

ON

MYOSIN LIGHT CHAIN KINASE

by

Karen Opleta

A Thesis

Submitted to the Faculty of Graduate Studies in

partial fulfillment of the requirements for the

degree of Master of Science

Department of Biochemistry

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Abstract

The generally accepted model for controlling smooth muscle contraction involves the phosphorylation of the 20,000 dalton light chain of myosin by a calcium-calmodulin dependent myosin light chain kinase, resulting in a form of myosin that can interact with actin. Dephosphorylation of myosin light chains by a phosphatase restores myosin to a form that cannot be activated. Myosin light chain kinase is in turn regulated through a dual mechanism of both the calcium concentration within the cell and adenylate cyclase-cAMP-protein kinase activity which phosphorylates the enzyme at two sites.

Myosin light chain kinase contains at least three functional domains: the catalytic domain containing the active site; the calmodulin binding domain; and two phosphorylation sites. Calmodulin binding to the kinase affects one of the phosphorylation sites, resulting in only one site being phosphorylated in the presence of calmodulin. Limited proteolysis using trypsin and chymotrypsin has shown that these three domains can be separated. After digestion of myosin light chain kinase by chymotrypsin, there is no longer any competition observed between protein kinase and calmodulin. Calmodulin binding to myosin light chain kinase causes a conformational change in the enzyme which appears to result in a masking of one phosphorylation site.

Abbreviations

ATP	Adenosine 5'-triphosphate
ATPγS	Adenosine 5'-0-(3-thiotriphosphate)
βME	2-mercaptoethanol
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N', tetraacetic acid
cAMP	Cyclic adenosine mono-phosphate
LBTI	Lima bean trypsin inhibitor
MLCK	Myosin light chain kinase
MLC	Myosin light chains
PAGE	Polyacrylamide gel electrophoresis
PK	Protein kinase
SDS	Sodium dodecyl sulfate
TPCK	L-1-Tosylamide-2-phenylethyl chloromethyl ketone
TLCK	Na-p-tosyl-L-lysine chloromethyl ketone
TEMED	N,N,N,N,-tetramethyl ethylenediamine
TRIS	Tris (hydroxy methyl) amino methane
PMSF	Phenylmethyl sulfonyl fluoride

Materials

Trypsin, chymotrypsin, lima bean trypsin inhibitor, soybean trypsin inhibitor, and pepstatin were obtained from Worthington. TPCK, TLCK, cAMP, 2-mercaptoethanol, SDS, DTT, EGTA, EDTA, Triton X-100, protein kinase, and sodium pyrophosphate were all obtained from Sigma.

ATP^{32} was bought from New England Nuclear. Acrylamide was bought from Bio-Rad. TEMED was obtained from Eastman Kodak. Sodium azide was bought from BDH Chemicals.

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Introduction

The calcium-calmodulin complex appears to be a universal regulator which regulates a large number of cellular activities and is not either tissue or species specific. cAMP has also been shown to be a universal messenger. Although it was thought that calcium was the main factor in regulating neurally responsive tissue and that cAMP was the second messenger in hormonally responsive tissue, it is becoming clear that both calcium and cAMP serve messenger funtions in both types of tissues;- calcium through calmodulin and cAMP through cAMP-dependent protein kinase. Many enzymes regulated by calmodulin are also substrates for cAMP-dependent protein kinase; - myosin light chain kinase is one of these enzymes. Phosphorylation of the kinase by protein kinase results in a 20 fold (Conti and Adelstein 1981) to 500 fold (Malencik et al 1982) decrease in affinity of the enzyme for calmodulin. This decrease in affinity of an enzyme for calmodulin after being phosphorylated by protein kinase is also seen in myelin basic protein, troponin I, and histone H2A with 2.5 to 5 fold decrease in affinity (Malencik et al 1982).

Myosin light chain kinase contains two phosphorylation sites which can be phosphorylated by cAMP-dependent protein kinase. However, if calmodulin is bound to the enzyme, only one site can be phosphorylated, indicating some form of competition between calmodulin and cAMP-dependent protein kinase (Conti and Adelstein 1980). Malencik et al (1982), through studies on various synthetic peptides, have suggested that modification of calmodulin binding sites may be a function of protein kinase, and that there is a similarity between calmodulin binding sites and sites recognized by protein kinase. To look more closely at this idea with respect to myosin light chain kinase, the kinase was subjected to proteolytic degradation. Attempts were then made to separate the calmodulin binding site from the phosphorylation sites and to try and establish the possible relationships between the two.

Smooth Muscle Contraction

General Overview

The ability of smooth muscles to convert chemical energy in the form of ATP into mechanical energy and movement is based on the coupling of ATP hydrolysis to a series of conformational changes in the myosin molecule, resulting in the attachment and disattachment of cross bridges between myosin and actin. In muscle, the only connections between myosin and actin are the cross bridges, which are actually the globular heads of the myosin molecule. In a contracted muscle, there is no change in the length of the thick and thin filaments, as it is only the angle change of the cross bridges that both generates and sustains muscle tension (Brading 1981).

In muscle excitation, the primary event is the release of bound calcium from internal sequestered calcium either from the sarcoplasmic reticulum or the plasma membrane. The calcium, mediated by calmodulin, activates myosin light chain kinase, which in turn alters the conformation of the myosin head through a phosphorylation mechanism. This allows the interaction of the cross bridges between myosin and actin. Although interaction occurs between the thick and thin filaments due to the phosphorylation and conformational change of the myosin component of the thick filaments, the structure of the thin filament may also be altered by the interaction. A co-operative process in which the initial contact of the cross bridges affects the remainder of non-attached cross bridges by modifying either the actin or tropomyosin in the thick filament may be involved in smooth muscle contraction, as during contraction only a small percentage of the cross bridges are attached at one time (Blaustein 1977).

Role of Calcium

The ability of electrically excitable smooth muscle cells to respond to changes in the external environment is dependent on their ability to maintain ionic gradients across the plasma membrane. Small

changes in permeability can result in significant movements of ions down electrochemical gradients. Ions entering the cell may initiate reactions within the cell themselves, or, alternatively, the ions may alter membrane potentials and cause further changes in permeability. Mechanisms for restoring the cell membrane to pre-stimulus levels in the form of energy requiring membrane pumps are also present in the cell membrane (Blaustein 1977).

Resting muscle cells have a free intracellular calcium concentration of about 10^{-7}M, with a rise in the concentration to 10^{-5}M resulting in muscle contraction (Szent-Gyorgyi 1975). Whether this increase in free calcium arises from an increased flux from external compartments or from the release of intracellular bound calcium, or both, has not as yet been conclusively shown.

Histochemical studies on calcium localization within the smooth muscle cells have shown that the sarcoplasmic reticulum is a main site for storage and release of calcium, along with the plasma membrane, mitochondria, and nucleus (Gabella 1981). Various types of smooth muscle differ in the time they are able to maintain contractile ability when incubated in an EGTA-containing solution (Kuriyama 1981). It has been inferred from this that smooth muscle cells differ either in the amount of stored calcium within the cell, or the degree of dependence on an extracellular influx of calcium as a triggering mechanism for contraction. The intracellular calcium may be closely associated with receptors; the activation of such receptors by endogenous agonists being the physiological stimulus for contraction. The interaction of an agonist with a membrane bound receptor may release internal calcium from binding sites so raising intracellular free calcium without causing a change in membrane potential. Alternatively, interaction with a membrane bound receptor may increase calcium influx through the opening of calcium and other ion channels. An increase in calcium influx in response to a neurotransmitter has been observed in some non-excitable muscles, but the influx appears to be too small to raise the intracellular calcium to a threshold level (Brading 1981). Calcium entering the cell may therefore act as a trigger for further release of calcium from internal stores.

There is indirect evidence supporting both a Na+:Ca++ exchange across the plasma membrane in smooth muscle cells (Blaustein 1977) and an energy dependent calcium extrusion pump (Brading 1981), but there is very little direct positive evidence for either. If these mechanisms do exist, they may be involved with the regulation of internal calcium concentrations, and in turn, the regulation of muscle contraction. Measurement of the influx and efflux of labelled calcium has shown that the majority of calcium exchanges rapidly, indicating some form of continual transmembrane movement (Brading 1981).

Structure of Smooth Muscle

Smooth muscle cells contain three types of filaments: thick filaments composed primarily of myosin; thin filaments composed primarily of actin; and intermediate filaments composed of a 55,000 dalton protein known as skeletin (Small and Sobieszek 1977). Tropomyosin is also associated with the thin filaments. The ratio of thick to thin filaments in vascular smooth muscle is 1:15 and in chicken gizzard 1:8 (Nonomura 1976).

Structure of Myosin

Myosin (MW 460,000) is a long, asymmetric molecule consisting of a globular head and a fibrous tail. The molecule contains one pair of heavy chains (MW 200,000) and two pairs of light chains that have varying molecular weights of 15,000 daltons to 27,000 daltons depending upon the species (Adelstein 1980). The amino terminal end of the heavy chain is globular while the carboxyl terminal end is fibrous. The fibrous end is in an α-helical conformation with the two chains wound around each other forming the myosin filament. The globular head, which is associated with the four light chains, binds to actin and contains the site of ATPase activity and appears to be required for the activity (Matsuda 1981). The second pair of light chains contain calcium-binding ability, are phosphorylated by myosin light chain kinase, and are involved in the actin-

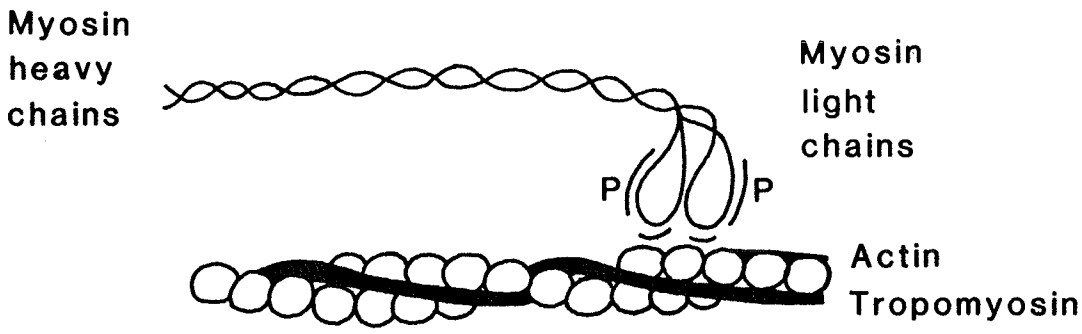


Figure 1. Diagram of the myosin, actin and tropomyosin arrangement in smooth muscle cells, (from Adelstein and Eisenberg 1980).

myosin interaction (Maita et al 1981).

Both the 20,000 dalton and 17,000 dalton light chain amino acid sequences have been elucidated in chicken gizzard (Maita et al 1981; Matsuda et al 1981). The serine residue that is phosphorylated in the 20,000 dalton regulatory light chain is located at residue 13, which is near the blocked amino terminus (Maita et al 1981). A large difference was observed between the 20,000 dalton light chains from smooth and skeletal muscle in the C-terminal regions, although a strong homology was observed in the calcium binding regions.

Structure of Actin

Actin (MW43,000) is a globular protein and the major constituent of thin filaments found in smooth muscle cells. X-ray diffraction studies have shown that fibrous actin is a double stranded αhelix of actin monomers. Each molecule of globular actin binds one calcium ion and one molecule of ATP followed by its polymerization into fibrous actin. The ADP formed by the splitting of the ATP remains bound to the fibrous actin chain. As well as having a structural function, actin serves to increase the rate at which myosin hydrolyzes ATP by interacting with the myosin globular heads to form an actin-myosin complex. Addition of ATP to actomyosin complex results in a dissociation of the complex into the actin and myosin (Adelstein and Eisenberg 1980).

Structure of Tropomyosin

Tropomyosin (MW 66,000) is composed of two helical subunits which form a two-chain twisted coil. Tropomyosin molecules are arranged end-to-end in the grooves of the actin filaments. The function of tropomyosin in smooth muscle is not clearly understood, although it does appear to increase the ATPase activity of purified myosin (Sobieszek and Small 1977).

Discovery of Myosin Light Chain Kinase

The regulatory role of calcium in all muscle tissue had been well established by the early 1970's, and it had been shown that in skeletal muscle the regulatory calcium sensitive proteins, troponin and tropomyosin, were found on the thin filament (Weber and Murray 1973). At first it was thought that a troponin-like mechanism existed in smooth muscle due to the presence of a troponin-C like protein (Head et al 1977). However, this protein was subsequently identified as calmodulin (Grand et al 1979) and researchers began looking at other regulatory mechanisms. It had been observed that phosphorylated myosin could be activated by actin resulting in the attachment of cross bridges between myosin and actin, and a muscle contraction. Dephosphorylation of the myosin light chain resulted in a form of myosin which could not interact with actin (Sobrieszek 1977). This evidence pointed to a calcium-dependent phosphorylation of the light chains which involved a calcium dependent regulatory kinase and a phosphatase.

Attempts to purify and characterize the kinase responsible for the phosphorylation of myosin light chains culminated in 1977 (Dabrowski et al). Before this time, only crude preparations were available, and it was impossible to determine just what the essential regulatory component was. Dabrowski (1977) took the supernatant obtained from the precipitation of tropomyosin and put it through a Sepharose 4B column. This resulted in three major fractions, two of which were required for activity of the kinase - a high molecular weight component of 105,000 and a low molecular weight of 17,000. The low molecular weight component was also shown to require calcium. The two fractions were then purified on a DEAE-cellulose column, and, using the purified preparations, Dabrowski et al (1977) clearly demonstrated the ability of the kinase to phosphorylate myosin light chains. He also showed a direct correlation between myosin phosphorylation and activation of ATPase activity. It was first suggested by Dabrowski et al (1977) that the calcium sensitivity of smooth muscle was due to the calcium requirement of the 17,000 low molecular weight protein. This protein was subsequently identified as calmodulin (Grand et al 1979). Later work by Adelstein and Klee (1981) showed that the kinase

was dependent upon calmodulin-calcium for activity, indicating the presence of a calmodulin binding site in the kinase.

Daniel and Adelstein (1976) also reported the purification of a myosin light chain kinase from human platelets, but with a lower molecular weight (80,000) and lacking a calcium requirement. This lack of a calcium requirement was probably due to the fact that the 80,000 kinase was a proteolytic product of a 105,000 kinase which was purified from the same source in 1979 by Hathaway and Adelstein, and which does have a calcium requirement. It has been well documented that limited proteolysis can result in a calcium independent form of myosin light chain kinase (Walsh et al 1979) due to the digestion of the calmodulin binding site, and it is probable that early reports of low molecular weight kinases were due to proteolysis during the purification procedure.

The major opposition to the phosphorylation-dephosphorylation mechanism was by Ebashi et al (1978), who held the opinion that regulation in smooth muscle operated via the proteins leiotonin A and C. According to Ebashi et al (1978), contraction of smooth muscle occurred whether or not the light chains were phosphorylated, but tropomyosin, leiotonin A (80,000 protein) and leiotonin C (18,000) were required for the activity. Also according to Mikawa et al (1978), although leiotonin C was calcium sensitive and calmodulin could replace its function in their actomyosin system, leiotonin C and calmodulin were different proteins. This observation was based on work which showed that leiotonin C could not replace calmodulin in a phosphodiesterase assay (Mikawa et al 1978). Leiotonin A was also shown to be different from myosin light chain kinase as leiotonin A could not phosphorylate myosin light chains (Mikawa et al 1978). At this point, no other researcher has confirmed Ebashi's findings, and the exact nature of leiotonin A and C and their role in smooth muscle contraction remains unclear.

The other possibility of a calcium regulatory role lay in the ability of myosin light chains to bind calcium, and it was thought by Chacks et al (1977) that calcium bound to myosin light chains was required for phosphorylation to occur. However, experiments by Kerrick and Hoar (1981) using adenosine 5'-0-(3-thiotriphosphate) (ATPγS) showed that if muscle fibers were pretreated with ATPγS, it would result in the myosin

becoming irreversibly thiophosphorylated. Myosin treated with ATPγS would then be active (contracted) even in the absence of calcium. This tended to support the phosphorylation mechanism, and also showed that calcium bound to myosin light chains was not required for contraction.

Most of the research has centered on the phosphorylation mechanism in smooth muscle contraction, and, in particular, on the role and structure of myosin light chain kinase.

Physical Properties of Myosin Light Chain Kinase

Molecular Weight

Myosin light chain kinase is a calcium-calmodulin dependent enzyme which catalyzes the transfer of γ-phosphate from ATP to the 20,000 dalton regulatory light chain of myosin. The enzyme has been isolated from skeletal (Yagi et al 1978), cardiac (Walsh et al 1979), and smooth (Adelstein and Klee 1981) muscle, with molecular weights ranging from 77,000 daltons for the skeletal muscle enzyme to 155,000 daltons for the bovine stomach enzyme (Table 1). Walsh et al (1983) have recently isolated a new form of myosin light chain kinase which can be separated from the 130,000 dalton enzyme by ion-exchange chromatography, and which contains polypeptide chains of 136,000 daltons and 141,000 daltons. Partial characterization of these peptides indicated that they specifically phosphorylated the 20,000 dalton light chain of myosin at the same site as the 130,000 dalton kinase and did not phosphorylate any other substrate. From these observations, Walsh et al (1983) suggests that the 130,000 kinase commonly seen may either be a proteolytic product of the 136,000 and 141,000 peptides, or they may be separate isoenzymes.

Cellular Location

Using a specific antibody against chicken gizzard myosin light chain kinase and indirect immunofluorescent microscopy, Guerriero et al (1981) have shown the kinase to be located on stress fibers and nucleoli of interphase cells; associated with the mitotic apparatus in dividing cells; bound to the myofibers in smooth muscles, and associated with the I band region of skeletal muscle.

Stress fibers in interphase cells have been shown to consist of actin, myosin, filamin, tropomyosin, αactinin, and calmodulin (Groschel-

Table 1

Molecular Weights of Myosin Light Chain Kinases from Various Sources

Molecular Weight	Source	Reference
77,000	rabbit skeletal	Nairn and Perry 1979
84,000	canine cardiac	Walsh et al 1979
105,000	chicken gizzard	Dabrowski 1977
130,000	turkey gizzard	Adelstein and Klee 1981
141,000 136,000	turkey gizzard	Walsh et al 1982
72,000	bovine cardiac	Walsh et al 1979
155,000	bovine stomach	Walsh et al 1982
105,000	human platelets	Hathaway and Adelstein 1979
105,000	BHK-21 cells	Yerna et al 1979
130,000	chicken gizzard	Uchiwa et al 1982

Stewart 1980), and the finding that myosin light chain kinase is also present indicates that contractility of the fibers and cell movement is controlled by a calcium activation of myosin ATPase.

The function of myosin light chain kinase in the nucleolus is unclear, but Guerriero et al (1981) suggest it may be connected with contractile proteins that have been reported to be in the nucleolus.

The movement of chromosomes is also controlled by an actin-myosin calcium-calmodulin complex, with calmodulin functioning both to activate myosin light chain kinase and to depolymerize microtubules.

The role of myosin light chain kinase in skeletal muscle may lie in the regulation of post-tetanic potentiation, rather than in the control of contraction, as it has been shown that phosphorylation of myosin light chains in skeletal muscle is not required for contraction (Manning and (Stull 1979).

Calmodulin Dependence of Myosin Light Chain Kinase

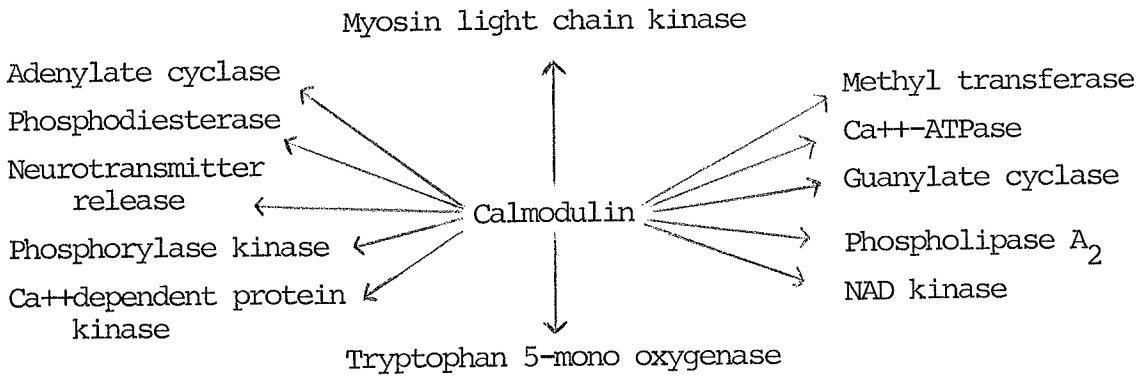
Discovery of Calmodulin

Calmodulin is a small (MW 17,000), heat stable, calcium binding protein found in all eukaryotic cells. In most cells, calmodulin is found both bound to various cellular structures as well as free in the cytosol.

Calmodulin was first recognized as a calcium requiring protein activator of bovine brain cAMP phosphodiesterase (Cheung 1970) and rat brain phosphodiesterase (Kakiuchi 1970) followed by erthrocyte Ca++-ATPase (Bond and Clough 1973) and brain adenylate cyclase (Brostrom et al 1975). The protein was referred to by many different names including modulator protein, activator protein, calcium-dependent regulator, troponin-C like protein, calcium dependent modulator protein, until 1978 when, by common agreement, the protein was named calmodulin (Cheung et al 1978).

General Features

Calmodulin is involved in a number of protein-protein interactions within the cell, as can be shown by the following diagram.



Calmodulin has been purified from a large variety of different sources including mammals (Teo et al 1973), lower vertebrates (Childers and Siegel 1975), invertebrates (Waisman et al 1975) and plants (Gomes et al 1979). The amino acid sequences of calmodulins from different sources have been found to be almost identical in vertebrates (Wang and Waisman 1979) with minor differences in amino acid composition in invertebrates and plants (Jamieson et al 1979; Yasawa et al 1980; van Eldick et al 1980). Sequence studies have shown calmodulin to be a member of a large family of calcium binding proteins which also include paravalbumin, troponin C, myosin light chains, and intestinal calcium binding protein (Kretsinger 1980). However, a single residue of trimethyllysine (residue 115) distinguishes calmodulin from other calcium binding proteins (Klee et al 1980).

Structure of Calmodulin

Calmodulin consists of 148 amino acid residues (Watterson et al 1980) and is composed of four homologous domains, each containing a helix-loop-helix structure that binds one calcium ion within the loop. The calcium binding loops include the residues 20-31, 56-67, 93-104, and 129-140 (Klee et al 1980). There is a large amount of homology between the four calcium binding domains, with the greatest amount between domains 1 and 3, and 2 and 4 (Watterson et al 1980). The loops contain amino acid sequences

favorable for the formation of β-turns (Vogt et al 1979). Calmodulin is a highly acidic protein with an isoelectric point of 3.9 - 4.3 (Klee et al 1980).

Calcium Binding to Calmodulin

Binding of calcium within the four calcium binding loops results in a conformational change and a 5-10% increase in the α-helix content of calmodulin (Wolff and Brostrom 1979). Binding studies have shown both negative co-operativity (Klee et al 1979) and positive co-operativity (Klee et al 1980) of the binding sites, depending on the calcium concentration and the methods of measurement. K_d values for calcium binding to calmodulin have been shown to be 10^{-6}M (Klee et al 1980), although the presence of a physiological concentration of Mg^{++} (10^{-3}M) and low ionic strength results in only 3 calcium binding to calmodulin.

Calmodulin Binding to Proteins

Interaction between calmodulin and target proteins occurs in at least two steps. The first step involves the binding of calmodulin with calcium which causes a conformational change within the calmodulin and results in an active form which can interact with target proteins. The second step involves the calcium-calmodulin complex binding to and either activating or deactivating the target protein (Kretsinger 1980). The number of calcium sites which must be occupied to activate either calmodulin or the target protein is not known at this time, and may be different for different proteins.

The question of why the amino acid structure of all calmodulins has been so highly conserved has interested many different researchers. One idea which has been put forward is that of a universal calmodulin binding site shared by all proteins which bind calmodulin. If such a site existed, it would restrict the 3-dimensional structure of calmodulin and thereby restrict the amino acid sequence. However, through the use of chemical modification techniques on calmodulin, Walsh et al (1978) have shown that phosphodiesterase and troponin C react with different areas on

the calmodulin molecule. This would indicate that different calmodulin binding proteins contain calmodulin binding sites which differ from one another. Each protein may then restrict only a certain area on the calmodulin; - but with different proteins all restricting a different area on the calmodulin, the end result would be the retention of the total amino acid structure of calmodulin.

It is also known that calmodulin contains four binding sites for calcium, and will undergo conformational changes as the sites become occupied (Wolff and Brostrom 1979). It has not been conclusively shown how many sites have to be occupied in order for calmodulin to bind to target proteins. It may be possible that different proteins recognize different conformational states of calmodulin depending on how many calcium sites are occupied.

Myosin Light Chain Kinase Binding to Calmodulin

Myosin light chain kinase is completely inactive in the absence of calcium or calmodulin and binds one mol kinase/mol calmodulin in the presence of calcium (Adelstein and Klee 1981). Due to the ability of calmodulin to bind calcium at four separate sites, two different mechanisms for the binding of calmodulin to myosin light chain kinase are possible. The first model involves a two-step mechanism with a calcium-calmodulin complex forming, followed by an interaction with the complex and the kinase, resulting in an active kinase. A second mechanism involves the binding of calmodulin with only some of the calcium sites occupied to the kinase, but with the enzyme remaining inactive until all four sites are occupied by calcium. Due to the strong affinity of myosin light chain kinase for the calcium-calmodulin complex, it has been suggested that the initial step in the inactivation of the kinase is the dissociation of calcium, rather than the dissociation of calmodulin, because of the rapid loss of enzyme activity upon addition of EGTA to an assay solution (Adelstein and Klee 1981).

It has been shown that the interaction of calmodulin with myosin light chain kinase produces a 30% increase in myosin light chain kinase tryptophan fluorescence, indicating a calmodulin-induced structural change in the enzyme (Johnson et al 1981). Using the method of changing

fluorescence, Johnson et al (1981) were able to examine the calcium dependence of the calmodulin-kinase binding, and also the effect of the ratio of calmodulin to kinase on tryptophan fluorescence. It was found that binding of the calmodulin to the kinase occurred as a single site was occupied by calcium on the calmodulin, but it was not shown whether the kinase was active in this state or not. It is inferred that calmodulin binding to the kinase produces structural changes in the kinase altering the state of some of its tryptophan residues. These findings were confirmed by Malencik et al (1982), although the changes they observed in the intrinsic fluorescence spectrum and the fluorescence polarization excitation spectrum of calmodulin binding to the kinase were smaller than those observed by Johnson et al (1981).

The optimal pH for calmodulin activation of myosin light chain kinase has been found to be between 6.0 and 7.5, a physiologically normal range (Blumenthal and Stull 1982).

Protein Kinase

Introduction

The activation of adenylate cyclase by hormonal action results in an increased level of cAMP and an activation of cAMP-dependent protein kinase. cAMP dependent protein kinase in turn catalyzes the transfer of the γ-phosphate group of ATP to either serine or threonine residues of protein substrates either activating or deactivating several target proteins within the cell.

The enzyme can be separated into two main fractions (type I and type II) by DEAE-chromatography. cAMP-dependent protein kinase (type I) was purified to homogeneity by Beavo et al (1974) from rabbit skeletal muscle, with type II first being obtained by Rubin et al (1972) from bovine heart. Both the type I and type II enzyme consists of two regulatory and two catalytic subunits, with the difference between the two types a result of different regulatory subunits. Activation of protein kinase by cAMP involves binding of one mole of kinase with two moles of cAMP to the regulatory subunits, with subsequent release of the catalytic subunits.

Substrate Specificity

cAMP-dependent protein kinase has been found in all mammalian species and is a secondary messenger for a large number of hormones (Table 2). The number of protein substrates for protein kinase is increasing rapidly as more systems are studied, and it is clear that two aspects are important for phosphorylation. First, the phosphorylation site must be both accessible to the enzyme, and second, it must contain a specific primary structure. The isolation and identification of the sites of phosphorylation of many proteins which are substrates of protein kinase has led to the observation that local primary structure of the substrates is an important determinant (Table 3). The increasing use of small, synthetic peptides as protein kinase substrates has shown that peptides as small as 5 or 6 residues can be good substrates for the enzyme, as long as the proper amino acid sequence is used (Table 4). Basic patterns are found in all protein kinase substrates. These include the presence of basic amino acid residues on the amino terminal side of the phosphorylation site with arginine residues predominating. Kemp (1976) has demonstrated the relative importance of arginine residues in protein kinase substrates through the use of synthetic peptides. The sequence ARG-GLY-TYR-SER-LEU-GLY can be phosphorylated by protein kinase, whereas the sequence GLY-GLY-TYR-SER-LEU-GLY can not be phosphorylated, the only difference between the two peptides being the presence or absence of the arginine residue. Further work by Kemp (1977) has also shown the relative importance of arginine residues, as substituting alanine, histidine, lysine, or homoarginine for arginine in heptapeptides resulted in an increase in the K_m values of the peptides of from 15 to 80 fold.

The phosphorylation site is usually serine, although the occasional threonyl residue is also phosphorylated as in myelin basic protein and protein phosphatase inhibitor (Carlston et al 1979). In natural protein substrates, two main patterns around the phosphorylation site are found. These are: 1) LYS-ARG-X-X-SER(P)
2) ARG-ARG-X-SER(P)

Hydrophobic residues are usually found on both sides of the phosphorylated serine (Krebs and Breavo 1979).

Table 2.

Hormones Using Camp as a Second Messenger

Calcitonin
Chorionic gonadontropin
Corticotropin
Epinephrine
Follicle-stimulating hormones
Glucagon
Luteninizing hormone
Lipotropin
Melanocyte-stimulating hormone
Norepinephrine
Parathyroid hormone
Thyroid stimulating hormone
Vasopressin

(from Bramson et al 1983)

Table 4.

Peptide Substrates for Protein Kinase

Peptide	$K_m$app (μM)	V_{max} (μmol/min/kg)
Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg	240	0.3
Arg-Gly-Tyr-Ser-Leu-Gly	4200±200	16.4±0.3
Gly-Gly-Tyr-Ser-Leu-Gly	12400±1300	0.4±0.02
His-Gly-Tyr-Ser-Leu-Gly	13400±500	0.9±0.02
Lys-Gly-Tyr-Ser-Leu-Gly	14700±400	0.8±0.01
Leu-Arg-Arg-Ala-Ser-Val-Ala	10	
Arg-Arg-Ala-Ser-Val-Ala	20	
Arg-Arg-Ala-Ser-Val	80	
Leu-Arg-Arg-Ala-Ser-Leu-Gly	16	20.0±0.05
Leu-Arg-Arg0Ala-Ser-Leu-Gly	4900±700	8.7±0.6
Leu-Arg-Ala-Ala-Ser-Leu-Gly	6300±400	5.3±0.2
Leu-Lys-Arg-Ala-Ser-Leu-Gly	1400±100	17.1±0.4
Leu-Arg-Lys-Ala-Ser-Leu-Gly	260± 10	16.9±0.3
Leu-His-Arg-Ala-Ser-Leu-Gly	415± 22	12.1±0.3

(from Bramson et al 1983)

Table 3.

Protein Substrates of cAMP-dependent Protein Kinase and the Sequences of Phosphorylatable Sites

Substrate	Sequence
Phosphorylase kinase (α subunit)	Ala-Arg-Thr-Lys-Arg-Ser-Gly-Ser(P)-Val-Tyr
Phosphorylase kinase (β subunit)	Phe-Arg-Arg-Leu-Ser(P)- Val-Asp-Thr-Ser-Ser-Leu
Glycogen synthase (site 1)	Lys-Ser-Asn-Ser(P)-Val-Asp-Thr-Ser-Ser-Leu
Glycogen synthase (site 2)	Lys-Arg-Ala-Ser(P)-Arg
Pyruvate kinase	Gly-Val-Leu-Arg-Arg-Ala-Ser(P)-Val-Ala-Glx
Histone H1	Ala-Lys-Arg-Lys-Ala-Ser(P)-Gly-Pro-Pro-Val
Histone $H2_a$	Ala-Lys-Thr-Arg-Ser(P)-Arg-Ala
Histone $H2_b$	Arg-Lys-Arg-Ser(P)-Arg-Lys-Glu-Ser(P)-Tyr-Ser
Troponin I	Val-Arg-Met-Ser(P)-Ala-Asp-Ala-Met Arg-Gln-His-Leu-Lys-Ser(P)-Val-Met
Myelin basic protein	Pro-Ser-Gln-Arg-His-Gly-Ser(P)-Lys-Tyr-Leu Gly-Val-Gly-Leu-Ser(P)-Leu-Ser-Arg

(from Carlson et al 1979)

The 3-dimensional configuration of protein substrates is also important in determining the substrate specificity although the actual configuration of the substrate as required by protein kinase is not known at this time. Granot et al (1983) have used NMR studies of various peptide substrates to look at the secondary structures, and have concluded that the structure required by protein kinase in its substrates is probably a coil, although a particular β-turn may be required.

Discovery of the Effect of Protein Kinase

Adelstein et al (1978) first reported that myosin light chain kinase could be phosphorylated by cAMP-dependent protein kinase. Although Adelstein et al (1978) only observed one mol phosphate incorporated into one mole of kinase, they also observed a 2-fold decrease in the rate at which the enzyme phosphorylated myosin light chains.

It has been known earlier that in smooth muscle, increased levels of cAMP inhibited tension development, but these results had been interpreted as cAMP effects on calcium transport and binding, and on energy utilization through glycolysis or lipolysis (Berridge 1975). With the knowledge that myosin light chain kinase was a substrate for protein kinase, Adelstein et al (1978) suggested a direct role for cAMP in regulating smooth muscle through the regulation of myosin light chain kinase.

Further work by Conti and Adelstein (1980) showed that the major effect of phosphorylation of myosin light chain kinase was to decrease the affinity of the enzyme for calmodulin, as it was found that the phosphorylated kinase had a lower specific activity at a given calmodulin concentration than did the unphosphorylated kinase. By 1981, it became apparent that myosin light chain kinase contained two phosphorylation sites, and that there existed a relationship between calmodulin binding and protein kinase phosphorylation. Conti and Adelstein (1981) found that when calmodulin was bound to the kinase, only one mole of phosphate could be incorporated per mole of kinase, and the activity of the enzyme was not affected. However, in the absence of calmodulin, two moles of phosphate could be incorporated, and a 10-20 fold increase in the amount of calmodulin required for 50% activation of the kinase was observed.

Tryptic digestion of the denatured kinase confirmed the presence of two phosphorylated sites (Conti and Adelstein 1981).

On the basis of their work with cAMP-dependent protein kinase and myosin light chain kinase, Conti and Adelstein (1980) put forward the idea of the link between hormonal binding to receptors and the subsequent relaxation of smooth muscle being due to the phosphorylation of myosin light chain kinase. Crucial to their mechanism of muscle relaxation was the idea that an equilibrium between unbound myosin light chain kinase and kinase bound to calmodulin must exist in the cell. Phosphorylation of unbound kinase would alter the equilibrium between active (kinase bound to calmodulin) and inactive (kinase not bound to calmodulin) kinase, resulting in an overall decrease in myosin light chain kinase activity (Figure 1A).

Comparison of Protein Kinase and Calmodulin Substrates

Through their use of small peptides, Malencik and Anderson (1982) have shown that there exist similarities in the amino acid sequences of peptides which bind calmodulin in a calcium dependent manner. The comparison of four different calmodulin binding peptides in Table 5 shows a strong homology in the amino acid sequences with a pattern of three strongly basic amino acid residues followed by a variable section of three amino acids followed by two hydrophobic residues. Malencik and Anderson (1982) have compared this pattern found in calmodulin binding proteins with that seen in cAMP-dependent protein kinase substrates, and have suggested that the similarity of the two patterns indicates that one of the functions of protein kinase may be to modify calmodulin binding sites. Further evidence to support this theory lies in the dual regulation of myosin light chain kinase and phosphorylase kinase by cAMP-dependent protein kinase and calmodulin. It has been shown that calmodulin bound to myosin light chain kinase will block one of the phosphorylation sites, indicating some form of competition between calmodulin and protein kinase. Also, phosphorylation of myosin light chain kinase by protein kinase increases the K_m of the kinase of calcium activation from 0.8 μM for the unphosphorylated kinase to 8 μM for the phosphorylated kinase (Rasmussen and Waisman 1983).

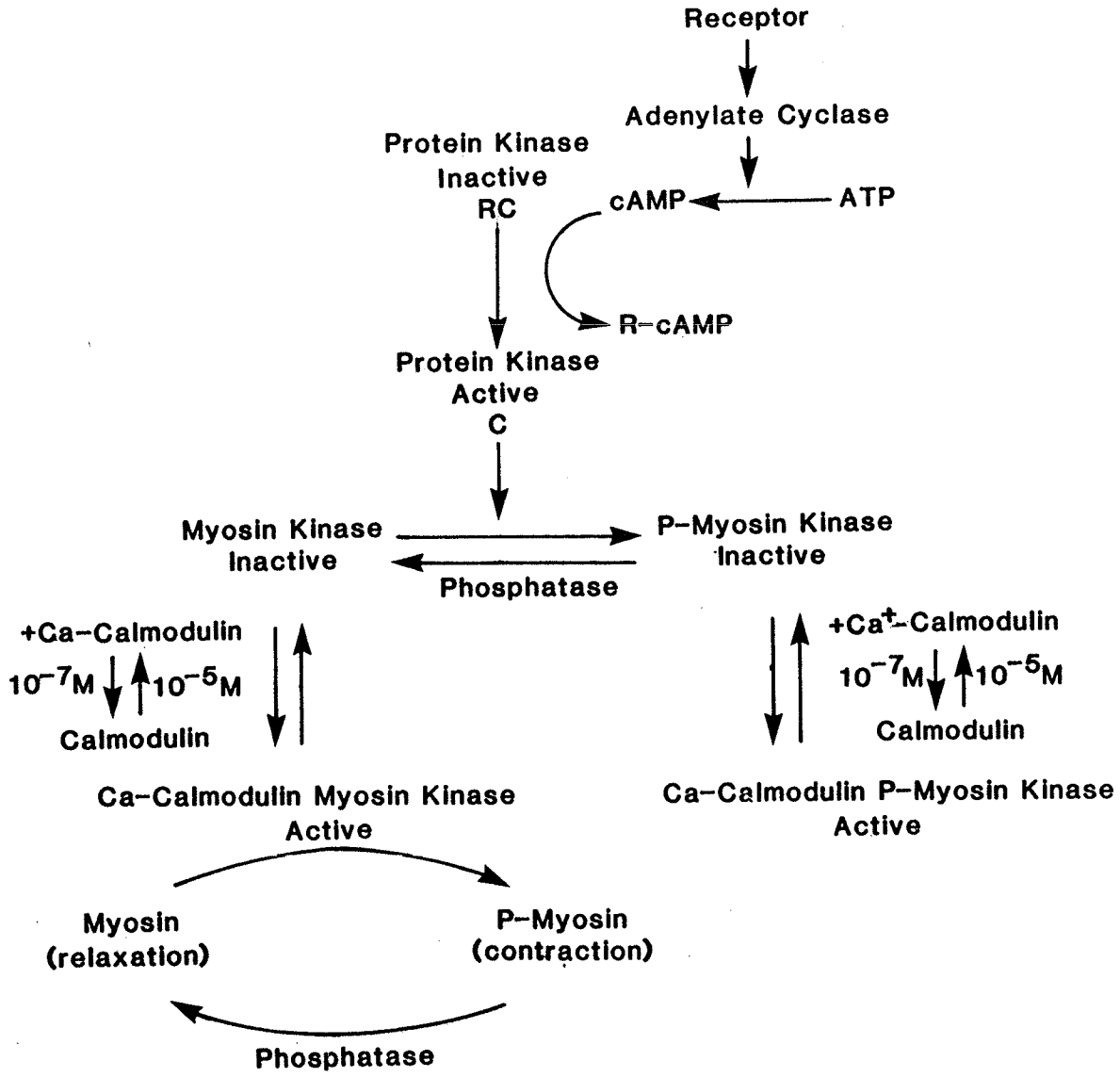


Figure 1A. Regulation of smooth muscle contraction by calcium and cAMP (from Adelstein and Eisenberg 1980).

Table 5

A Comparison of Protein Kinase substrates with Calmodulin Binding Peptides[1]

Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met	Substance P
Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Glucagon (16-26)
Lys	Arg	Arg	Pro	Val	Lys	Val	Tye	Pro	Asn	Gly	ACTH (16-26)
Lys	Lys	His	Ala	Asn	Lus	Ile	Ile	Ala	Ash	Lys	B-Endorphin (29-19)
Lys	Arg	Lys	Glu	Ile	Ser	Val	Ala	Gly	Leu		Synthetic substrate
Thr	Lys	Arg	Ser	Gly	Ser	Val	Tye	Glu	Pro	Leu	Phosphorylase kinase
Arg	Lys	Glu	Ser	Tye	Ser	Val	Tyr	Val	Tyr		Histone $H2_b$
Arg	Gln	His	Leu	Lys	Ser	Val	Met	Glu	Leu		Troponin 1
Val	Arg	Arg	Ser	Asp	Arg	Ala	Tyr	Ala			Cardiac Troponin L
Gln	Arg	His	Gly	Ser	Lys	Tyr	Leu	Ala			Myelin Basic Protein (8-16)

Basic Hydrophobic

[1] The first four amino acid sequences are from calmodulin binding proteins; the second four sequences represent phosphortable sites from protein kinase substrates (from Malencik and Anderson 1982)

To explain the calmodulin and protein kinase competition for myosin light chain kinase, several mechanisms have been proposed. One mechanism involves the binding of calmodulin to one of the actual phosphorylation sites, physically masking the site from protein kinase access. Another mechanism involves the binding of calmodulin to a site close to one of the phosphorylation sites and the resultant conformational change causing a portion of the molecule to swing out and mask the phosphorylation site from protein kinase access. Initial studies by Walsh et al (1982) indicate the second mechanism to be more likely for myosin light chain kinase.

Structure of Myosin Light Chain Kinase

Through the use of hydrodynamic and CD spectroscopic techniques on both the native enzyme and proteolytic products, Mayr and Heilmeyer (1983) have produced a structural model for skeletal muscle myosin light chain kinase. The model consists of a head region which shows a large content of α-helix and contains the active site and the calmodulin binding site, and a tail region which is asymmetric and which has a high content of Pro, Gly, Ala, and Glx (Figure 2). Mayr and Heilmeyer (1983) suggest that the tail region may contain either a βsheet or a collagen like helix, and may function to anchor the enzyme to myofilaments. This may be the case, as Walsh et al (1982) have shown that myosin light chain kinase will bind to actin.

Proteolytic Studies

Trypsin Results

The results obtained from characterizing the enzyme would indicate that myosin light chain kinase contains at least three domains; - a calmodulin binding domain (Adelstein and Klee 1981), an active site, and two sites phosphorylated by protein kinase (Conti and Adelstein 1980). Myosin light chain kinase may also contain a site which binds to actin as preliminary results by Walsh et al (1982) indicate that gizzard kinase binds to actin under conditions similiar to those in vivo.

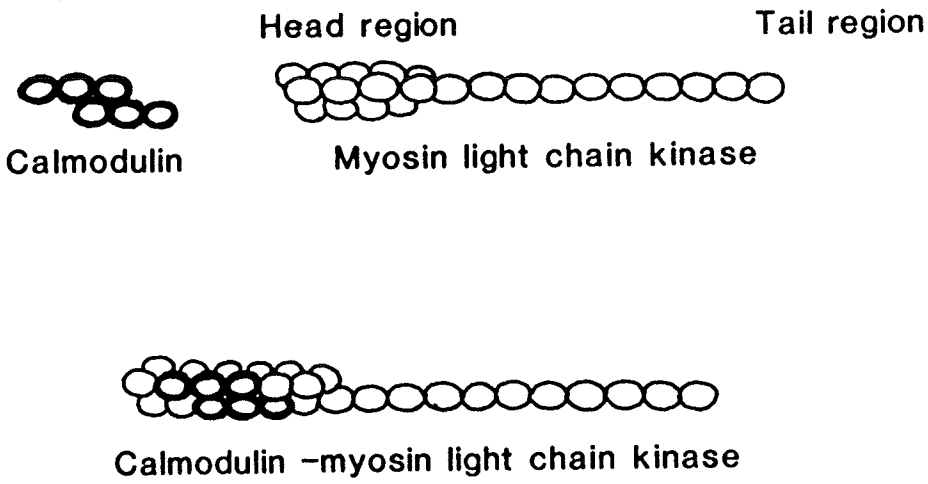


Figure 2. A structural model of skeletal muscle myosin light chain shown bound to calmodulin, (from Mayr and Heilmeyer 1983).

To try and show that the three domains of myosin light chain kinase are distinct from each other and can be separated by proteolytic digestion, Walsh et al (1980) phosphorylated the enzyme with the catalytic subunit of cAMP dependent protein kinase, subjected the phosphorylated enzyme to limited tryptic digestion, and applied the digest to a calmodulin affinity column. Three peaks were observed coming off the column - the first peak which did not bind to the column contained all the radioactivity. It consisted of three peptides of 30,000, 22,000, and 15,000 daltons, with the 22,000 dalton peptide containing all the radioactivity. The second peak was non-specifically bound to the column and was washed off using 0.8M NaCl. It consisted off a major peptide of 55,000 daltons which showed slight kinase activity. The third peak consisted of two peptides of 50,000 and 24,000 daltons which bound to the affinity column and which were washed off using an EGTA buffer. Walsh et al (1980) concluded from this that the phosphorylation sites and the calmodulin binding domain are distinct from each other, as all the phosphate was recovered in the 22,000 dalton peptide, and it was separate from the 50,000 dalton peptide and the 24,000 dalton peptide which bound to the calmodulin-affinity column. However, it was not until 1981 that Conti and Adelstein showed that myosin light chain kinase contained two phosphorylation sites, and Walsh et al (1980) did not show that in their digest, the kinase was phosphorylated at two sites. No attempt was made to phosphorylate any of the peptides to see if any further phosphate could be incorporated, so it is possible that the kinase was phosphorylated at only one site. Also, it is well known that many basic peptides will bind to calmodulin well. It is possible that the 50,000 dalton and 24,000 dalton peptides which bound to the calmodulin-affinity column did not contain the actual calmodulin binding site, but instead had predominantly basic residues.

Conti and Adelstein (1981) showed that limited tryptic digestion of the myosin light chain kinase-calmodulin complex yielded a 20,000 dalton phosphorylated peptide similiar to that seen by Walsh et al (1980). However, when phosphorylation was carried out in the absence of bound calmodulin, a total of 1.4 to 2.0 mol phosphate/mol kinase was incorporated and tryptic digestion of the denatured kinase resulted in two phosphorylated peptides, one of which co-electrophoresed with the P^{32} peptide released from the other digest. Tryptic digest of the native enzyme resulted in the release of a single phosphorylated peptide.

Chymotrypsin Results

The pattern of chymotrypsin digestion of myosin light chain kinase is dependent on the presence or absence of calmodulin and calcium. If calmodulin is present, and the digest is carried out on a kinase-calmodulin complex, a calcium-insensitive kinase of 80,000 is produced (Walsh et al 1982). The specific activity of the 80,000 enzyme was found to be 6.5±0.2 μmol P/min/mg in the presence of calcium, and 8.3±0.3 μmol P/min/mg in the absence of calcium. Walsh et al (1982) attributed this loss of calcium sensitivity to the loss of the calmodulin binding domain, as the 80,000 peptide would not bind to a calmodulin affinity column. The phosphorylation sites were also absent, as no P^{32} could be incorporated into the 80,000 peptide. However, if the entire digest was treated with the catalytic subunit of cAMP dependent protein kinase, a maximum amount of phosphate (1.4-2.0 mol) was incorporated into a 23,000 piece whether or not calmodulin was present. Because the presence of calmodulin did not affect the incorporation of phosphate into the 23,000 peptide, it would appear that once the kinase has been digested, calmodulin bound to the kinase no longer affects the phosphorylation reaction.

Walsh et al (1982) also found that if calmodulin was not bound to the native enzyme during digestion, a 95,000 peptide resulted from a limited chymotrypsin digest, which retained its calcium sensitivity. Because the pattern of chymotrypsin digestion is dependent on the presence or absence of calmodulin, it would appear that calmodulin binding to the kinase alters the conformational state of the enzyme so exposing some chymotrypsin sensitive sites to the protease.

General Experimental Procedures

Electrophoretic Procedures

SDS-PAGE

Proteins were examined by 0.1% sodium dodecyl sulfate, 7.5-20% polyacrylamide gradient slab gel electrophoresis according to Laemmli (1970). The following solutions were used:

Solution	Composition
A	30 g acrylamide 0.8 g MBA H_2O to 100 ml
B	1.5 M Tris-HCl pH 8.8
C	10% SDS
D	0.5 M Tris-HCl pH 6.8
E (running buffer)	3 g Tris-HCl 14.4 g glycine 1 g SDS H_2O to 1000 ml pH 8.3
F (sample buffer)	0.05 M Tris-HCl pH 6.8 1% SDS 0.01% Bromophenol Blue 30% glycerol Made 1% in β-mercaptoethanol just before use

The seperating gel (7.5-20%) was prepared by mixing the following solutions.

7.5%	-	Solution A	7.5 ml
		Solution B	7.5 ml
		H_2O	12.9 ml
		Solution C	0.6 ml
		Ammonium persulfate (12 mg/ml)	1.5 ml
		TEMED	0.04 ml

20%	-	Solution A	20 ml
		Solution B	7.5 ml
		H_2O	-
		Solution C	0.6 ml
		Ammonium persulfate (12 mg/ml)	1.5 ml
		TEMED	0.04 ml

The stacking gel (5% acrylamide, 0.13% MBA) was prepared by mixing the following solutions.

Solution A	5.0 ml
Solution B	7.5 ml
H_2O	15.7 ml
Solution C	0.3 ml
Ammonium persulfate (8 mg/ml)	1.5 ml
TEMED	0.02 ml

Protein samples were dissolved in solution F (sample buffer) and immersed in a boiling water bath for 2 minutes. Electrophoresis was performed at 25-35 mamps. Gels were stained overnight with 0.25% coomassie brilliant blue G-250 in methanol:acetic acid:H_2O (5:1:5) and subsequently destained electrophoretically with 7.5% acetic acid.

Autoradiography

Film

Kodak X-Omat AR film

Solutions

Kodak liquid X-ray developer and replenisher
Kodak rapid fixer with hardener

Procedure

Gels containing proteins labelled with P^{32} were dried and placed into Kodak X-Omatic cassettes with Kodak X-Omat AR film. To develop the autoradiogram, the film was placed in the developer for 5 minutes (in the darkroom) followed by 2 minutes in fixer. The film was then washed for 5 minutes in flowing water and hung to dry.

Assay of Myosin Light Chain Kinase

Myosin light chain kinase activity was assayed by measuring the amoung of acid-stable phosphate incorporated into the myosin light chain using Mg+(γ-P) ATP as the phosphate donor in the presence and absence of calcium and calmodulin.

Reagents

Total volume: 100 µl

50 mM Tris-HCl pH 7.6
10 mM Mg+Ac
10 mM βME
0.1 mM $CaCl_2$ or 1 mM EGTA
10 µg/ml calmodulin
5 mg/ml mixed light chains
2 µg/ml MLCK
0.6 mM ATP - (γ P)ATP diluted with 20 mM stock non-radioactive ATP, pH 7.6 to give an activity of 1000 cpm/pmol ATP.

Reaction Mixture

Buffer (500 mM Tris 7.6, 100 mM MgAc, 100 mM βME, 0.1% Tween)	10 µl
$CaCl_2$/EGTA (1 mM/10 mM)	10 µl
Calmodulin (100 µg/ml)	10 µl
Mixed light chains (10 mg/ml)	10 µl
MLCK (20 µg/ml)	10 µl
ATP	10 µl

Procedure

Pre-incubate for 2 minutes at 25°C.

Add ATP to start the reaction.

To stop reaction, 20 µl of the reaction mixture was removed and spotted on to filter discs and put through the following washing procedure:

a) 10 minutes in 10% TCA and 2% sodium pyrophosphate (cold)
b) 30 minutes in 10% TCA (cold)
c) 20 minutes in 5% TCA (room temperature)
d) 20 minutes in 5% TCA (room temperature)
e) 10 minutes in 95% ethanol

The filter paper discs were then dried and counted in the scintillation counter.

Assay of cAMP-dependent Protein Kinase

The activity of cAMP-dependent protein kinase was assayed by measuring the amount of acid-stable phosphate incorporated into an acceptor protein substrate, using $Mg^{+}(\gamma P)ATP$ as the phosphate donor.

Reagents

Total volume: 100 µl

30 mM Tris-HCl pH 7.5
10 mM MgCl
0.4 mM EGTA
10 µg/ml protein kinase
0.6 mM ATP - $(\gamma P^{32})ATP$ diluted with 20 mM stock non-radioactive ATP pH 7.6 to give an activity of 1000 cpm/pmole ATP
10 µM MLCK

In some experiments, 10 µg/ml calmodulin was added to give a final concentration of 10 µmol either in the presence of 0.2 mM $CaCl_2$ (over EGTA) or 0.4 EGTA.

Reaction Mixture

Buffer (300 mM Tris-HCl 7.6, 100 mM MgCl, 0.1% Tween)	10 µl
$CaCl_2$/EGTA (6 mM/4 mM)	10 µl
Calmodulin (100 µg/ml)	10 µl
Protein kinase (100 µg/ml)	10 µl
MLCK	50 µl
ATP	10 µl

Procedure

Pre-incubate for 2 minutes at 25°C.

Add ATP to start the reaction.

To stop reaction, 20 µl of the reaction mixture was removed and spotted on to filter discs and put through the following washing procedure:

a) 10 minutes in 10% TCA and 2% sodium pyrophosphate (cold)
b) 30 minutes in 10% TCA (cold)
c) 20 minutes in 5% TCA (room temperature)
d) 20 minutes in 5% TCA (room temperature)
e) 10 minutes in 95% ethanol

The filter paper discs were then dried and counted in the scintillation counter.

Preparation of Myosin Light Chains

Myosin light chains were prepared according to the method of Perrie and Perry (1970) and the regulatory light chain by the method of Nairn and Perry (1979). The following procedures were carried out at 4°C unless otherwise stated.

Preparation of Myofibrils

Chicken gizzards (fresh or stored frozen at -20°C) were cleaned of fat and surrounding tissue, chopped and ground to yield 250 g of mince. The mince was washed twice for 15 minutes each time by stirring in 5 volumes of 15 mM Tris-HCl pH 7.5, 60 mM KCl, 5 mM 2-mercaptoethanol, 10 μM $CaCl_2$, 0.5% Triton X-100 (v/v) and centrifugation at 4500 g for 10 minutes after each wash. The mince was washed a third time with the same buffer without Triton X-100 and centrifuged at 4500 g for 10 minutes.

Extraction of Myosin

The myofibrillar pellet was extracted with 3 volumes of 0.15 M potassium phosphate pH 6.5, 10 mM pyrophosphate for 6 hours with continuous stirring, and centrifuged at 4500 g for 20 minutes. The supernatant was dialyzed against 15 mM Tris-HCl pH 7.5, 5 mM 2-mercaptoethanol, 2 mM EDTA (2x8 liters) and then against 8 liters of the same buffer containing 1 mM $NaHCO_3$. Centrifugation was then carried out at 4500 g for 20 minutes and the pellet dried overnight.

Preparation of Light Chains

Four grams of myosin were dissolved in 250 ml of 5 guanidine-HCl 10 mM 2-mercaptoethanol and the pH adjusted to 7.0. After 3 hours of stirring, centrifugation was carried out at 10,000 g for 15 minutes, and the pellet discarded. 250 ml of cold H_2O was added to the supernatant, followed by 1 liter of cold ethanol. After mixing for 15 minutes, centrifugation was carried out at 10,000 g for 15 minutes and the pellet discarded. The supernatant was taken to dryness on a rotary evaporator

and then dialyzed overnight against 5-6 changes (8 liters each) of 10 mM NH_4HCO_3, 10 mM 2-mercaptoethanol,the last change being without 2-mercaptoethanol. Centrifugation was carried out at 10,000 g for 15 minutes, and the pellet discarded. The supernatant was lyophilized.

Purification of Regulatory Light Chain

The lyophilized light chains were dissolved in a minimum volume of 50 mM Tris-HCl pH 8.5, 15 mM 2-mercaptoethanol, and 5 M urea (buffer A). and applied to a column (2.5x90 cm) of DEAE-Sephacel pre-equilibrated with buffer A. Excess protein was washed off the column with buffer A and bound proteins were eluted with a linear gradient (0-0.3 M KCl) made from 1 liter of 50 mM Tris-HCl pH 7.6, 15 mM 2-mercaptoethanol, 5 M urea and 1 liter of 50 mM Tris-HCl pH 7.6, 15 mM 2-mercaptoethanol, 5 M urea and 0.3 M KCl. Fractions were examined by 0.1% SDS 7.5-20% polyacrylamide gradient slab gel electrophoresis and active fractions were pooled and lyophilized.

Purification of Myosin Light Chain Kinase

Myosin light chain kinase was isolated and purified by a modification of the procedure described by Walsh et al (1983). The procedure is the same except for the inclusion of phenylmethyl sulfonyl fluoride (PMSF) in all buffers to prevent proteolysis. The following procedures were performed at 4° C unless otherwise stated. Table 9 shows a summary of the procedure.

Homogenization

Chicken gizzards (fresh or stored frozen at -20°C) were cleaned of fat and surrounding tissue, chopped, and ground to yield 250 grams of tissue. The tissue was homogenized in a Waring blender at top speed for 3X5 seconds in 4 volumes of 20 mM Tris-HCl pH 7.5, 40 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.05% (v/v) Triton X-100, 0.1% PMSF, and centrifuged at 15,000 g for 15 minutes. The pellet was suspended and homogenized as before in 4 volumes of the same buffer only with Triton X-100 and centrifuged again. The pellet was resuspended and homogenized again in 4 volumes of the same buffer without Triton X-100 and centrifuged. The homogenate supernatant was run on an SDS-polyacrylamide gel (7.5-20%) (lane 1 - Figure 3).

Kinase Extraction

The pellet was suspended and homogenized in 4 volumes of 40 mM Tris-HCl pH 7.5, 60 mM KCl, 25 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1% PMSF, and centrifuged at 15,000 g for 30 minutes. The supernatant was then filtered through glass wool and analyzed by SDS-PAGE (lane 2 - Figure 3).

Ammonium Sulfate Fractionation

Solid ammonium sulfate was added with stirring to the filtered supernatant to 60% saturation (363 grams/liter). Buffer containing 40 mM Tris-HCl pH 7.5, 60 mM KCl, 25 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT was then added to reduce the ammonium sulfate concentration to 40% saturation (226 grams/liter). The supernatant was then centrifuged at 15,000 g for

Table 9.

Purification of Myosin Light Chain Kinase from chicken gizzard[2]

Fraction	Volume (ml)	Protein[3] (mg/ml)	Specific Activity (μmol/mg)	Yield (%)	Purification
Mg^{++}-extract supernatant	1096	3.4	0.024	100	-
40-60% ammonium sulfate	44	8.4	0.26	89	8.9
Dialyzed Affi-Gel Blue eluate	76	0.98	0.92	62	31.7
DEAE-Sephacel eluate	54	0.26	3.4	44	117.0

[2] Starting material was 250 grams chicken gizzard

[3] Determined according to Bradford, M. Anal. Biochem. 72, 248 (1976).

30 minutes. After centrifugation, the supernatant was filtered through glass wool and ammonium sulfate was added to 60% saturation (123 grams/liter). The sample was centrifuged at 15,000 g for 30 minutes. The supernatant was discarded and the pellet was dissolved in approximately 50 ml of 20 mM K_2HPO_4 pH 8.0, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.02% NaN_3, 0.1% PMSF (buffer D) with the aid of a hand operated glass-glass homogenizer and dialyzed overnight vs buffer D (2x10 liters).

Affi-Gel Blue Chromatography

The dialyzed sample (lane 3 - Figure 3) was applied to a column (1.5x25 cm) of Affi-Gel Blue (Bio-Rad) previously equilibrated with buffer D. Excess protein was washed off with buffer D, and bound proteins were eluted with a linear NaCl gradient of 200 ml each of buffer D and buffer D containing 0.08 M NaCl. Fractions were assayed for myosin light chain kinase activity in the presence and absence of Ca^{++} and examined by 0.1% SDSpolyacrylamide gradient slab gel electrophoresis (7.5-20%) (Figure 4). Fractions were pooled on the basis of their activity and the gel pattern, and dialyzed overnight vs 2x10 liters of 20 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1% PMSF (buffer E).

Assay of Myosin Light Chain Kinase

Myosin light chain kinase activity was assayed by measuring the amount of acid-stable phosphate incorporated into the myosin light chain using $Mg^{+}(\gamma\text{-}P^{32})$ ATP as the phosphate donor in the presence and absence of calcium and calmodulin. The total volume of the incubation mixture was 100 µl and included 10 µl of buffer (50 mM Tris-HCl pH 7.6, 10 mM MgAc, 10 mM βME, 0.1% Tween), 10 µl $CaCl_2$ or 10 µl EGTA, 10 µl 10 µg/ml calmodulin, 50 µl 5 mg/ml mixed light chains, 10 µl ATP, and 10 µl of kinase sample. The samples were pre-incubated for 2 minutes at 25°C, with ATP added to start the reaction. To stop the reaction, 20 µl of the reaction mixture was removed and spotted on to filter paper discs, and put through the washing procedure of TCA followed by ethanol. The filter paper discs were dried and counted in the scintillation counter.

NOTICE/AVIS

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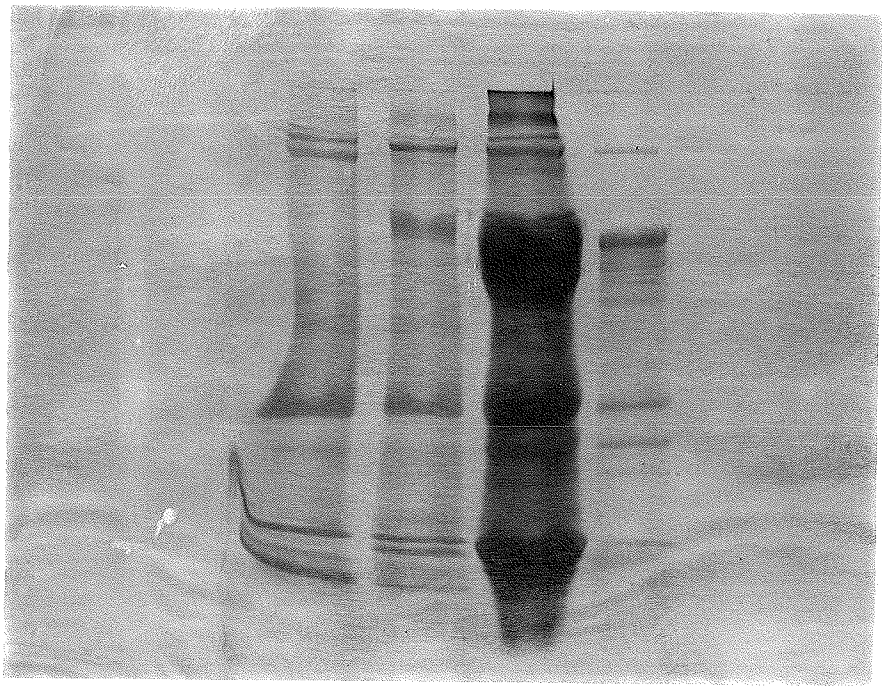
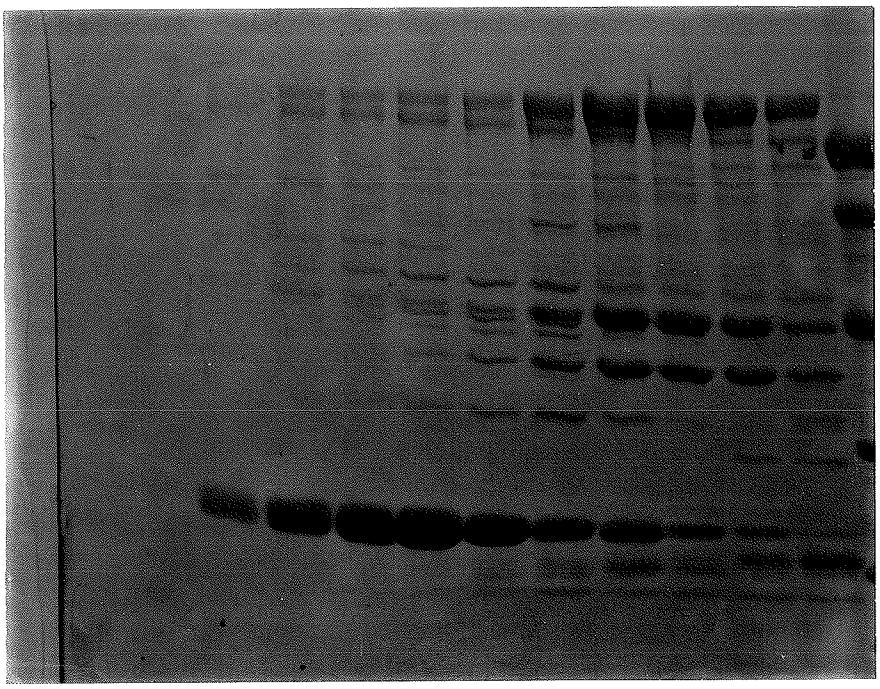


Figure 3.

Purification of myosin light chain kinase. Lane 1 represents the homogenate supernatant from the first three washes; lane 2 is the kinase extraction; lane 3 is the dialyzed ammonium sulfate cut applied to the Affi-Gel Blue column; lane 4 is the dialyzed eluate from the Affi-Gel blue column applied to the DEAE-column.

An SDS-polyacrylamide 7.5-20% gradient slab gel stained with coomassie-blue.



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Lanes 35 45 50 55 65 71 77 83 90 (Fractions from Affi-Gel Blue column)

Figure 4.

An SDS-polyacrylamide 7.5-20% gradient slab gel stained with coomassie-blue of fractions from the Affi-Gel Blue column.

The following fractions appear on the gel (left to right): 35, 45, 50, 55, 65, 71, 77, 83, 90. Fractions 67-88 were pooled and dialyzed.

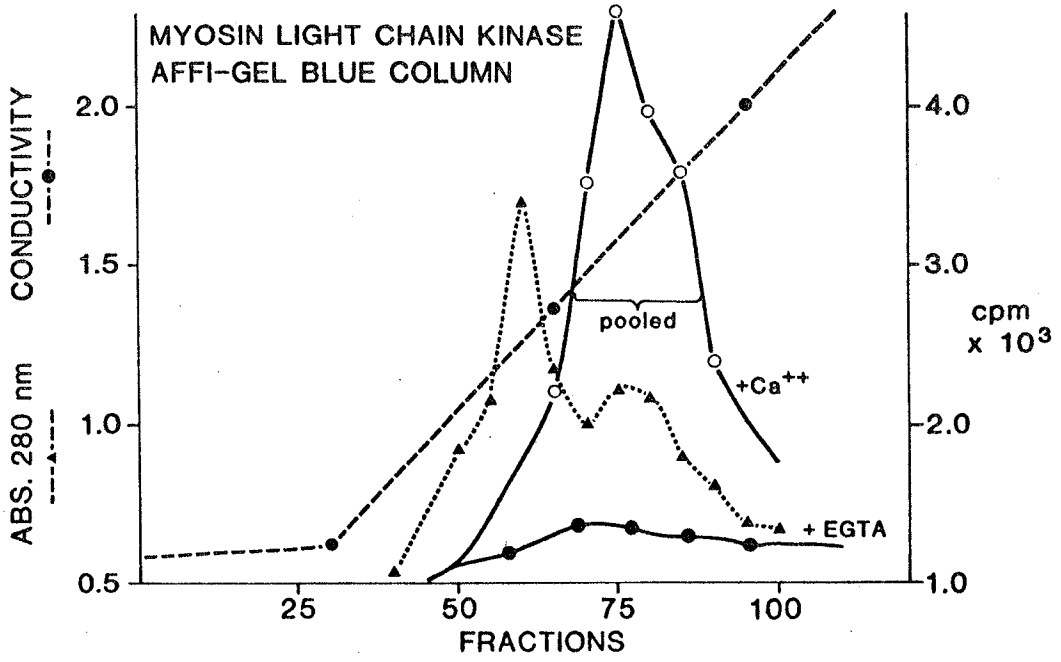

Figure 5.

Affi-Gel Blue chromatography of the dialyzed 40-60% ammonium sulfate fraction. The dialyzed sample was loaded on a column (1.5 x 25 cm) of Affi-Gel Blue previously equilibrated with Buffer D. Excess protein was washed off with Buffer D and bound proteins were eluted with a linear NaCl gradient of 200 ml each of Buffer D and Buffer D containing 0.8M NaCl.

MLCK activity was assayed in the presence of 0.1 mM $CaCl_2$ (—O—) or 1 mM EGTA (—●—). Fractions (4ml) were collected at a flow rate of 20 ml/hr. Fractions indicated were pooled and dialyzed prior to ion-exchange chromatography.

DEAE-Sephacel Chromatography

The dialyzed sample pooled from the Affi-Gel blue column was applied to a column (1.5 x 25 cm) of DEAE-Sephacel previously equilibrated with Buffer E. Excess protein was washed off the column with Buffer E and bound proteins were eluted with a linear NaCl gradient of 250 ml each of Buffer E and Buffer E containing 0.3M NaCl (Figure 6). Fractions were assayed for myosin light chain kinase activity in the presence and absence of calcium and examined by 0.1% SDS polyacrylamide gradient slab gel electrophoresis (7.5-20%) (Figure 7). Fractions were pooled on the basis of their activity measurements and the gel pattern, made 5% w/v in sucrose and stored at -70°C. All further experiments were done within two weeks of freezing the kinase, as the kinase tended to degrade upon storage.

Amino Acid Composition

An amino acid analysis was performed on the purified kinase (Table 6) and is shown as compared to the values as determined by Adelstein and Klee (1981).

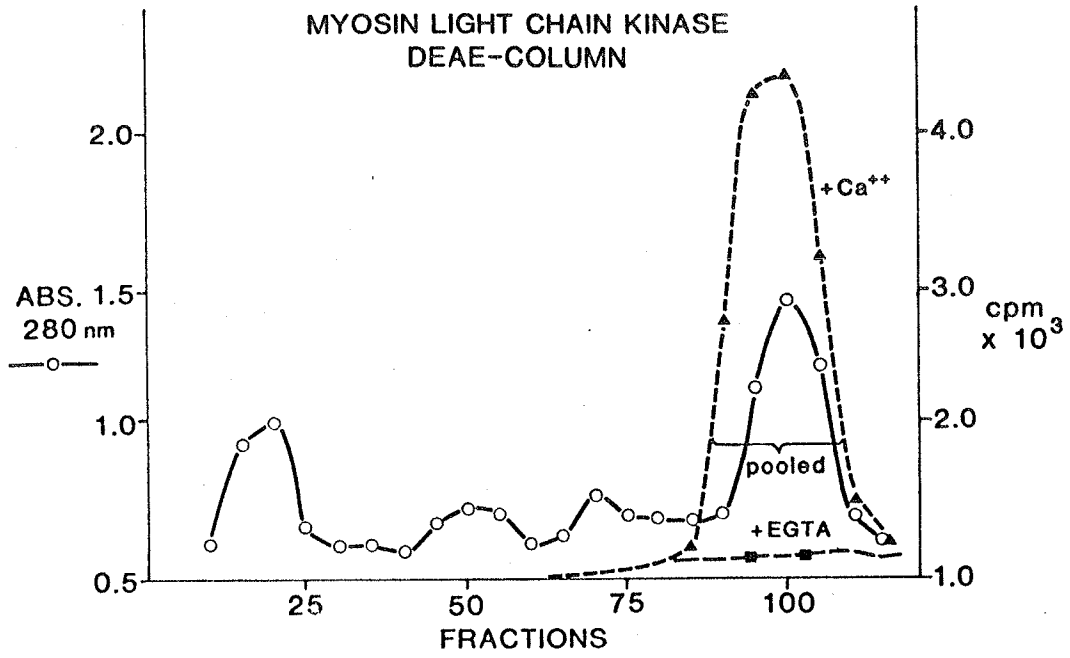

Figure 6.

DEAE-Sephacel ion-exchange chromatography of myosin light chain kinase. The dialyzed eluate from the Affi-Gel Blue column was loaded on a column (1.5 x 25 cm) of DEAE-Sephacel previously equilibrated with Buffer E. Excess protein was washed off the column with Buffer D, and bound proteins were eluated with a linear NaCl gradient of 250 ml each of Buffer E and Buffer E containing 0.3M NaCl.

MLCK activity was assayed in the presence of 0.1 mM $CaCl_2$ (—▲—) or 1 mM EGTA (—■—). Fractions (4 ml) were collected at a flow rate of 20 ml/hr. Fractions indicated were pooled and stored as described.

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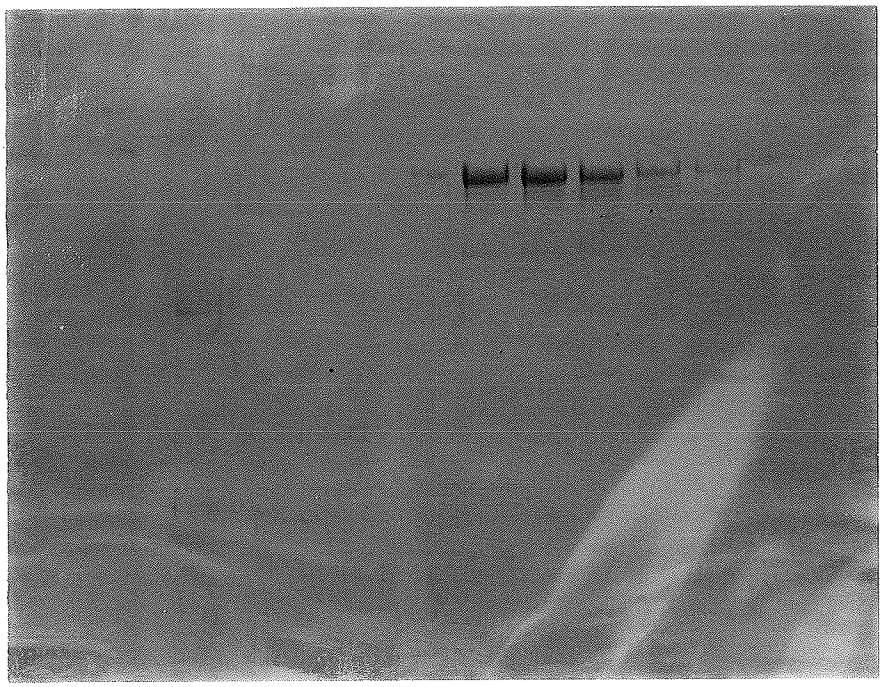

--- 130,000

Lane 80 85 90 100 105 110 (Fractions from DEAE-Sephacel column.)

Figure 7.

An SDS-polyacrylamide 7.5-20% gradient slab gel stained with coomassie-blue of fractions from the DEAE-Sephacel column.

The following fractions appear on the gel (left to right): 80, 85, 90, 100, 105, 110. Fractions 87-107 were pooled, dialyzed and stored frozen at -20°C.

Table 6

Purification of Myosin Light Chain Kinase

Amino Acid Composition[2]

Amino Acid	Concentration (nmoles)	Composition	Theoretical[3]
Lysine	110.64	100	102
Histidine	13.95	12	16
Arginine	50.96	46	38
Aspartic Acid	107.61	97	92
Threonine	64.55	59	61
Serine	74.25	67	86
Glutamic Acid	138.36	125	139
Proline	54.06	49	47
Glycine	58.87	53	71
Alanine	72.68	66	73
Valine	71.58	65	52
Methionine	43.27	28	20
Isoleucine	53.26	49	44
Leucine	87.39	73	79
Tyrosine	25.39	23	23
Phenylalanine	30.61	27	27
Cysteic Acid	16.0	14	-

[3]Values taken from the amino acid composition as determined by Adelstein and Klee (1981) for chicken gizzard based on Mr of 105,000

[2]Amino acid composition of chicken gizzard myosin light chain kinase based on a M_r of 125,000

Trypsin Digest

Introduction

Myosin light chain kinase was subjected to limited trypsin digestion to see if the calmodulin binding domain, the phosphorylation sites, and the active site can be separated from one another. If the calmodulin binding domain is different from both phosphorylation sites, it may be possible to separate these two functional areas using trypsin. If two peptides can be obtained - one of which will bind calmodulin, and the other which protein kinase will phosphorylate at two sites - then it will indicate that these two functional areas are not the same residues. Both the phosphorylated and unphosphorylated form of the kinase were digested. The phosphorylated enzyme was assayed to ensure that a maximum amount of phosphate (1.5-1.8 mol phosphate/mol kinase) was incorporated into the enzyme. After being subjected to tryptic digestion, the phosphorylated kinase digest was applied to a calmodulin affinity column. Theoretically, the peptide containing the calmodulin binding site should bind to the affinity column and be separated from all other peptides. If phosphorylation and calmodulin binding to the kinase are physically blocking each other in some way, then after digestion releases the peptide containing the two phosphorylation sites, the peptide containing the calmodulin binding site should also be exposed, and bind to the column. If however, the same residues are involved in both calmodulin binding and phosphorylation, then digestion should not result in a peptide containing the calmodulin site as well as a peptide containing two phosphorylation sites.

Digests were also carried out in the presence of calmodulin and calcium to see if calmodulin binding to the kinase would affect the pattern of cleavage.

Methods

Phosphorylation

Myosin light chain kinase (10^{-12} M final concentration) was incubated with 10 µg/ml of the catalytic subunit of cAMP dependent protein kinase, 0.6 mM (γ-^{32}P)ATP (178 cpm/pmol), 50 mM Tris-HCl pH

7.6, 10 mM MgAc, 10 mM βME, 0.2 mM EGTA for 1 hour at 24°C. Phosphate incorporation was measured by removing 20 µl of the incubation mixture and spotting onto filter paper discs. The discs were washed, dried, and counted in a scintillation counter. Phosphorylation (1.5-1.8 mol phosphate/mol kinase) of the kinase was confirmed by SDS-polyacrylamide gels followed by autoradiography.

Digestion

Either 50 µl of phosphorylated myosin light chain kinase labelled with P^{32} or 50 µl of unphosphorylated myosin light chain kinase was incubated with trypsin (1 mg /ml) at a protease:protein weight ratio of 1:500 in 20 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, at 24°C. For digestion of the kinase-calmodulin complex, $CaCl_2$ and calmodulin were added to a final free concentration of 0.5 mM. Digestion was carried out for times ranging from 10 seconds to 1 hour, with the digestion being stopped by the addition of 10 µl of TPCK (1 mg/ml). 35 µl of the digestion mixture was then added to SDS buffer, immediately immersed in boiling water for 2 minutes, and applied to SDS 7.5-20% gradient polyacrylamide gels.

Affinity Chromatography

The tryptic digest of P^{32} labelled myosin light chain kinase was applied to a column (3x20 cm) of calmodulin-Sepharose prepared by the method of Klee and Krinks (1978) and pre-equilibrated with 20 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT (Buffer A). The column was washed with the same buffer, then with buffer A containing 0.2 M NaCl to remove non-specifically bound peptides. Finally, those peptides which bound to the column were eluted with buffer A plus 0.2 M NaCl and 2 mM EGTA. Fractions of 3 ml each were collected at a flow rate of 20 ml/hr, with samples from each tube run on SDS gels. Autoradiograms were performed on the gels to locate the P^{32} labelled peptides. Attempts were made to phosphorylate the remaining fractions that did not show any P^{32} labelling, as well as to try and incorporate any further phosphate into the already labelled peptides.

Aliquots of 50 µl were taken from each tube and incubated with 10 µg/nl of the catalytic subunit of cAMP dependent protein kinase for 1 hour as described previously.

Results

Digestion of myosin light chain kinase in an EGTA buffer by trypsin to ensure no calmodulin was bound to the enzyme resulted in a major band at 58,000; a band at 32,000; one at 28,000; and 2 bands at 18,000 and 15,000 (Figure 9). The kinase-calmodulin complex was subjected to an identical digest with no change in the cleavage pattern (Figure 10). The kinase was not phosphorylated in either of these digests. It would appear that calmodulin binding to the kinase does not result in any trypsin sensitive residues becoming exposed.

Digestion of myosin light chain kinase which had been phosphorylated by protein kinase (1.7 mol phosphate/mol kinase) resulted in a similiar cleavage pattern (Figure 11) to that seen in the previous digests (Figures 9 and 10) on unphosphorylated kinase. The disappearance of the band at 58,000 in Figures 9 and 10 as compared to Figure 11 is a result of the longer digestion times in those digests. In Figure 11, the digestion times go from 10 seconds in lane 2 to 2 minutes in lane 5, while in Figures 9 and 10, the digestion times go from 10 seconds in lane 2 to 30 minutes in lane 6. The autoradiogram performed on the gel in Figure 11 showed all the phosphate located in the 28,000 peptide (Figure 12). The band phosphorylated under the main kinase band probably represents a proteolytic product of the kinase, as it has been well documented that kinase stored in the freezer will develop several bands in a short time (Walsh et al 1980).

The phosphorylated kinase (1.7 mol phosphate/mol kinase) was subjected to trypsin digestion for 10 minutes and then the entire digest was applied to a calmodulin affinity column (Figure 14). The peptides coming off the column in peak 1 included all the peptides which did not bind to the column and washed off with the loading wash. All of the applied P^{32} was located in this wash indicating that the 28,000 peptide did not bind to the calmodulin affinity column. The peptides coming off the column in peak 11 included all the peptides which were bound non-specifically to the column, as this peak was washed off with a high

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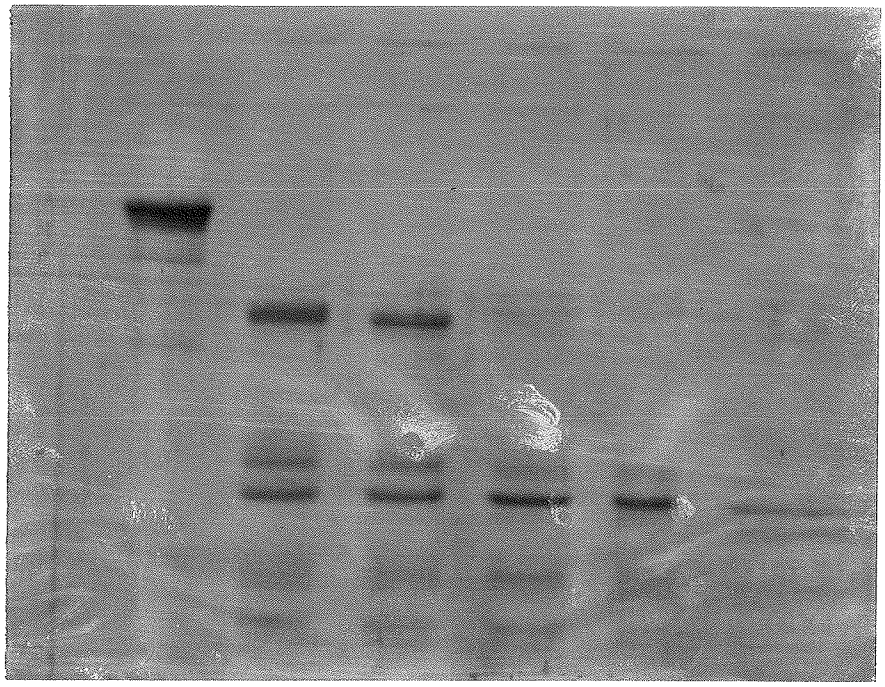

Figure 9.

Trypsin digest of myosin light chain kinase run on a 7.5-20% gradient SDS-polyacrylamide slab gel and stained with coomassie blue.

Lane 1 represents undigested kinase; the following lanes are increasing times of digestion: 30 seconds, 1 minute, 5 minutes, 10 minutes, 30 minutes.

Lane 1 - myosin light chain kinase
2 - 30 seconds digestion
3 - 1 minute digestion
4 - 5 minute digestion
5 - 10 minute digestion
6 - 30 minute digestion

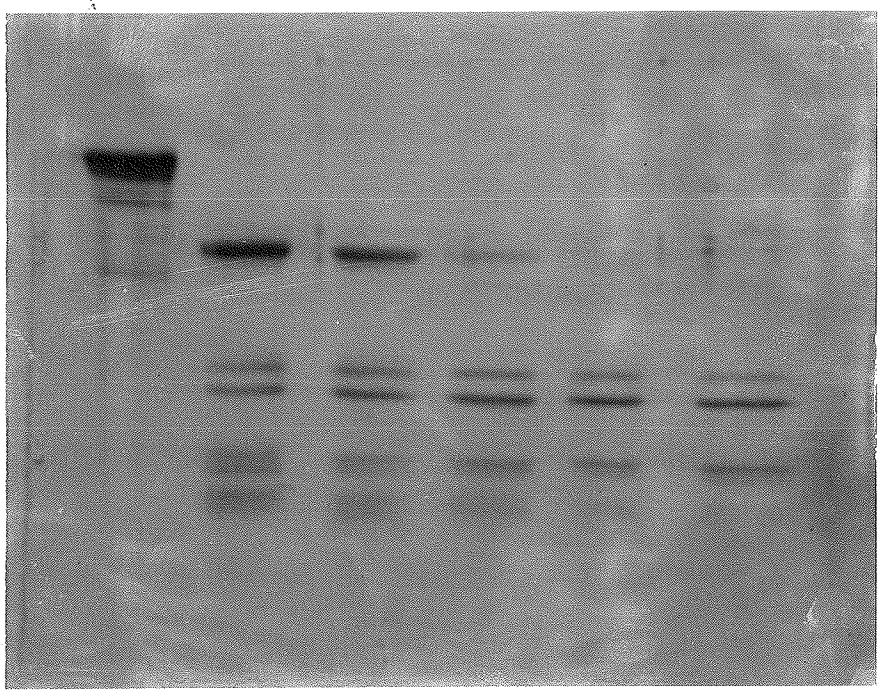

Figure 10.

Trypsin digest of myosin light chain kinase-calmodulin complex run on a 10% SDS-polyacrylamide slab gel and stained with coomassie blue.

Lane 1 represents undigested kinase; the following lanes are increasing times of digestion: 30 seconds, 1 minute, 5 minutes, 10 minutes, 30 minutes.

Lane 1 - myosin light chain kinase
2 - 30 second digestion
3 - 1 minute digestion
4 - 5 minute digestion
5 - 10 minute digestion
6 - 30 minute digestion

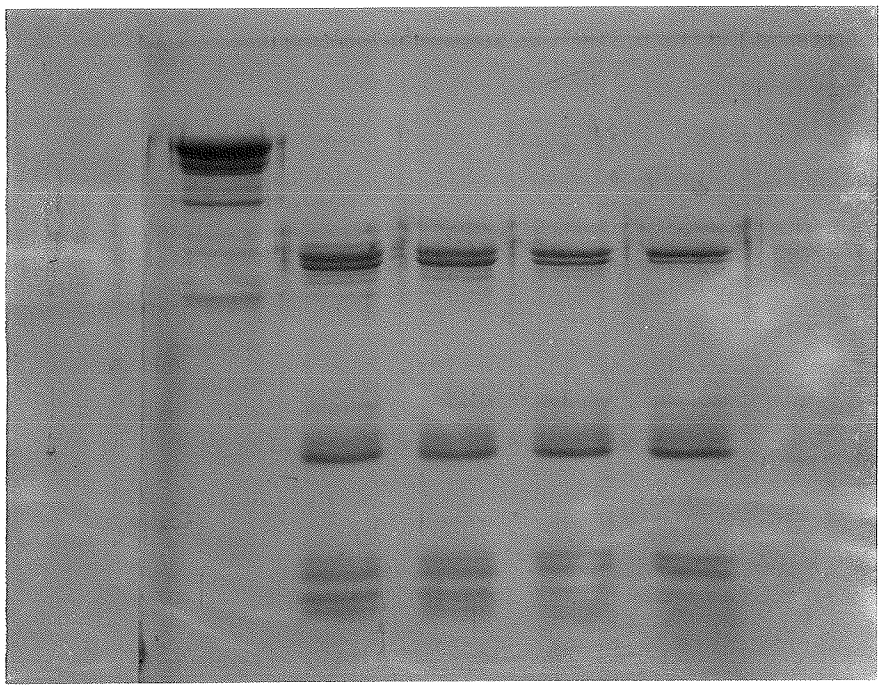

Figure 11.

Trypsin digest of phosphorylated myosin light chain kinase run on a 7.5-20% gradient SDS-polyacrylamide slab gel and stained with coomassie blue.

Lane 1 represents undigested kinase; the following lanes are increasing times of digestion: 10 seconds, 30 seconds, 1 minute, 2 minutes.

Lane 1 - myosin light chain kinase
2 - 10 second digestion
3 - 30 second digestion
4 - 1 minute digestion
5 - 2 minute digestion

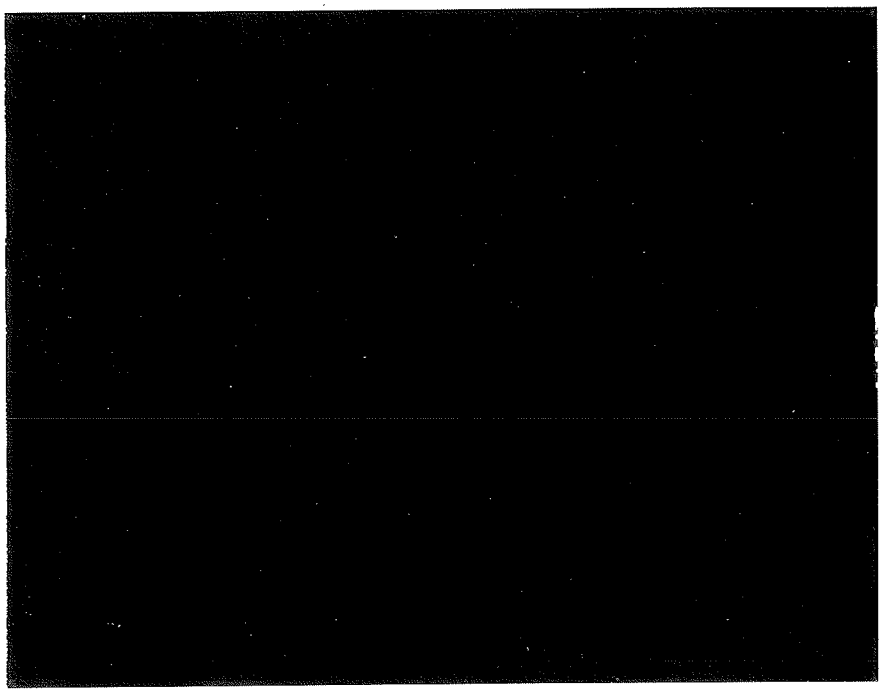

Figure 12.

An autoradiogram on the trypsin digest of phosphorylated myosin light chain kinase (Figure 9).

The digest was run on a 7.5-20% SDS-polyacrylamide gradient slab gel, the gel was dried, and the autoradiogram performed. Lane 1 represents undigested kinase; the following lanes are increasing times of digestion - 10 seconds, 30 seconds, 1 minute, 2 minutes.

Lane 1 - myosin light chain kinase
2 - 10 second digestion
3 - 30 second digestion
4 - 1 minute digestion
5 - 2 minute digestion

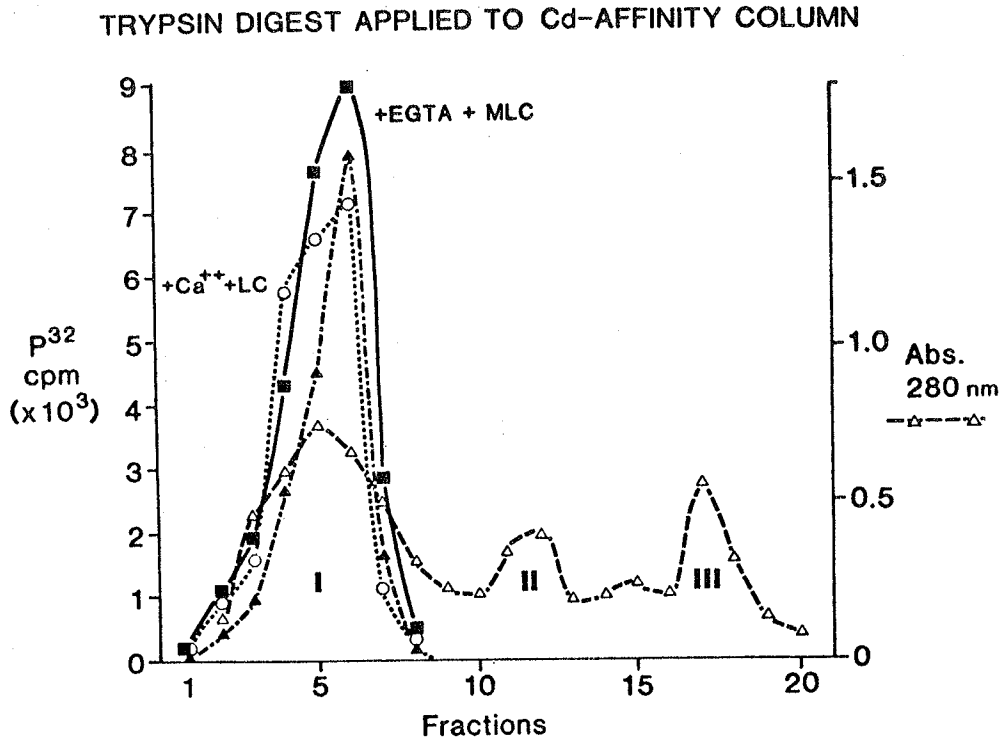

Figure 14.

The tryptic digest of myosin light chain kinase was applied to a calmodulin -affinity column (3 x 20 cm) pre-equilibrated with Buffer A. Excess protein was washed off the column with Buffer A (peak I). Peptides which bound non-specifically to the column were eluated with Buffer A containing 0.2M NaCl (peak II). Those peptides which bound to the column were eluated with Buffer A containing 0.2M NaCl and 2 mM EGTA (peak III). Fractions (3 ml) were collected at a flow rate of 20 ml/hr.

MLCK activity was assayed in the presence of 0.1 mM $CaCl_2$ (--0--) or 1 mM EGTA (—■—). Fractions were also counted in a scintillation counter to locate the previously labelled P^{32} peptides (--▲--).

NaCl buffer. The 32,000 and 18,000 peptides were seen in this wash. The 15,000 peptide was found in the third peak and was washed off the column with an EGTA and NaCl buffer, indicating that this peptide did bind to the calmodulin located in the column. Due to the small amount of material applied to the column, the gel which was run on the fractions was stained very faintly and is not included in this thesis.

Comparing these results to those seen by Walsh et al (1980) shows a difference in the molecular weights of peptides binding to the column. Walsh et al (1980) observed a 50,000 peptide and a 24,000 peptide binding to the column, as compared to a 15,000 peptide in this digest. It is possible that one or more of these peptides was binding non-specifically to calmodulin on the column, as it has been well-documented that calmodulin will bind many different basic proteins (Malencik and Anderson 1982). It is also possible that the 15,000 dalton peptide is a proteolytic product of either the 24,000 dalton peptide or the 50,000 peptide seen by Walsh et al (1980). It is also possible the 24,000 dalton peptide is a proteolytic product of the 50,000 dalton peptide, and that all three peptides contain the calmodulin binding domain. Further work will have to be done to determine which peptide actually contains the calmodulin binding site.

When the peptides coming off the column were incubated with protein kinase and ATP, no further phosphate could be incorporated into any of the fractions. If the kinase was maximally phosphorylated before digestion, then it was expected that no further phosphorylation would be possible. It is not clear why 2 mol phosphate/mol kinase is not always seen with maximal phosphate incorporation, but it is possible that some of the kinase may already contain some phosphate when isolated. It is also possible that either the kinase preparation or the light chain preparation may be contaminated with a phosphatase which would remove the phosphate group from the kinase.

It would appear from this digest that the calmodulin binding domain is located on a peptide which is separate from the peptide containing the two phosphorylation sites, and that the two phosphorylation sites can not be separated from each other by a limited trypsin digest on native kinase.

Chymotrypsin Digest

Introduction

As the results obtained from the trypsin digest indicated that the phosphorylation sites could be separated from the calmodulin binding domain on myosin light chain kinase, the kinase was subjected to chymotrypsin digestion. It has been reported that the cleavage pattern of the kinase-calmodulin complex is different from that of the kinase alone (Walsh et al 1982) so initial digests were performed to confirm this observation. As in the trypsin digests, the cleavage patterns of the phosphorylated kinase and the unphosphorylated kinase were compared.

Methods

Phosphorylation

Myosin light chain kinase (10^{-12} M final concentration) was incubated with 10 µg/ml of the catalytic subunit of cAMP dependent protein kinase, 0.6 mM (γ-^{32}P)ATP (178 cpm/pmol), 50 mM Tris-HCl pH 7.6, 10 mM MgAc, 10 mM βME, 0.2 mM EGTA for 1 hour at 24°C. Phosphate incorporation was measured by removing 20 µl of the incubation mixture and spotting onto filter paper discs. The discs were washed, dried, and counted in a scintillation counter. Maximum phosphorylation of the kinase (1.5-1.8 mol phosphate/mol kinase) was confirmed by SDS-polyacrylamide gels.

Digestion

Either 50 µl of phosphorylated myosin light chain kinase labelled with P^{32} or 50 µl of unphosphorylated kinase was incubated with chymotrypsin (1 mg/ml) at a protease:protein weight ratio of 1:500 in 20 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, at 24°C. For the digest of kinase-calmodulin complex, $CaCl_2$ and calmodulin were added to a final free concentration of 0.5 mM.

Digestion was carried out for times ranging from 10 seconds to 1 hour, with the digestion being stopped by the addition of either 10 µl of lima bean trypsin inhibitor (1 mg/ml) or TLCK (1 mg/ml). 35 µl of the digestion mixture was added to SDS buffer, immediately immersed in boiling water for 2 minutes, and applied to SDS 7.5-20% polyacrylamide gradient gels.

Results

As shown by Walsh et al (1982), calmodulin has an effect on chymotrypic digestion of myosin light chain kinase. If the digest is carried out on a kinase-calmodulin complex, a major band appears at 92,000, with a second band appearing at 60,000, and three minor bands at 32,000, 28,000, and 26,000. If the digest is carried out in an EGTA buffer to prevent calmodulin from being bound to the kinase, a major band appears at 84,000, with the same pattern of minor bands - 60,000, 32,000, 28,000, and 26,000 (Figure 15).

The kinase was then phosphorylated by protein kinase, both in the presence and absence of calmodulin. In the absence of calmodulin, the kinase had a phosphate content of 1.8 mol phosphate/mol kinase. Digestion of the kinase then resulted in all the phosphate being released in a 28,000 peptide (Figure 16 - lanes 2-9). In the presence of calmodulin, the kinase had a phosphate content of 0.7 mol phosphate/ mol kinase. Digestion of the kinase-calmodulin-P^{32} labelled peptide also resulted in all of the phosphate being released in the same 28,000 peptide (Figure 16 - lanes 12-18. As phosphorylation of the kinase does not affect either the trypsin or the chymotrypsin digestions, it probably does not significantly alter the conformation of the kinase. The binding of calmodulin to the kinase however, must cause a conformational change in the enzyme resulting in the exposure of chymotryptic sensitive residues. Figure 17 shows a comparison of the chymotryptic and tryptic cleavage patterns of free myosin light chain kinase, with the reaction stopped by the addition of lima bean trypsin inhibitor. Table 7 summarizes both the trypsin and chymotrypsin digestion results.

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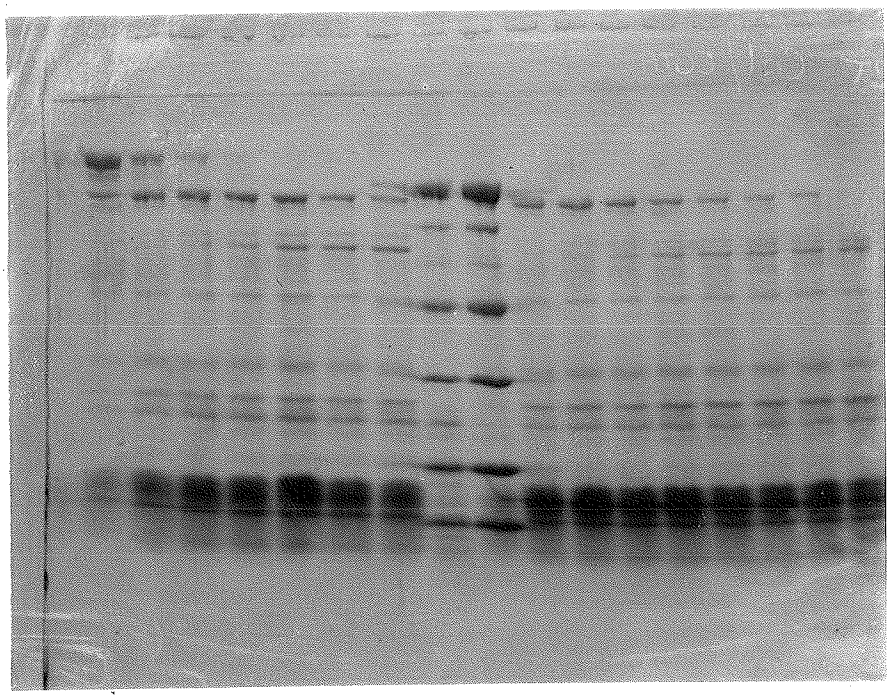


Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 15.

A comparison of the chymotryptic digest of myosin light chain kinase and myosin light chain kinase-calmodulin complex.

Lane 1 - undigested kinase
2 - CT digest of kinase-calmodulin complex - 10 seconds
3 - CT digest of kinase-calmodulin complex - 30 seconds
4 - CT digest of kinase-calmodulin complex - 1 minute
5 - CT digest of kinase-calmodulin complex - 2 minutes
6 - CT digest of kinase-calmodulin complex - 3 minutes
7 - CT digest of kinase-calmodulin complex - 5 minutes
8 - MW markers
9 - MW markers
10 - CT digest of kinase - 10 seconds
11 - CT digest of kinase - 30 seconds
12 - CT digest of kinase - 1 minute
13 - CT digest of kinase - 2 minutes
14 - CT digest of kinase - 3 minutes
15 - CT digest of kinase - 5 minutes
16 - CT digest of kinase - 7 minutes
17 - CT digest of kinase - 10 minutes.

Lime bean trypsin inhibitor was used to stop the reaction.

Molecular weight markers - Phosphorylase b - 94,000
Albumin - 67,000
Ovalbumin - 43,000
Carbonic anhydrase - 30,000
Trypsin Inhibitor - 20,100
Lactalbumin - 14,400

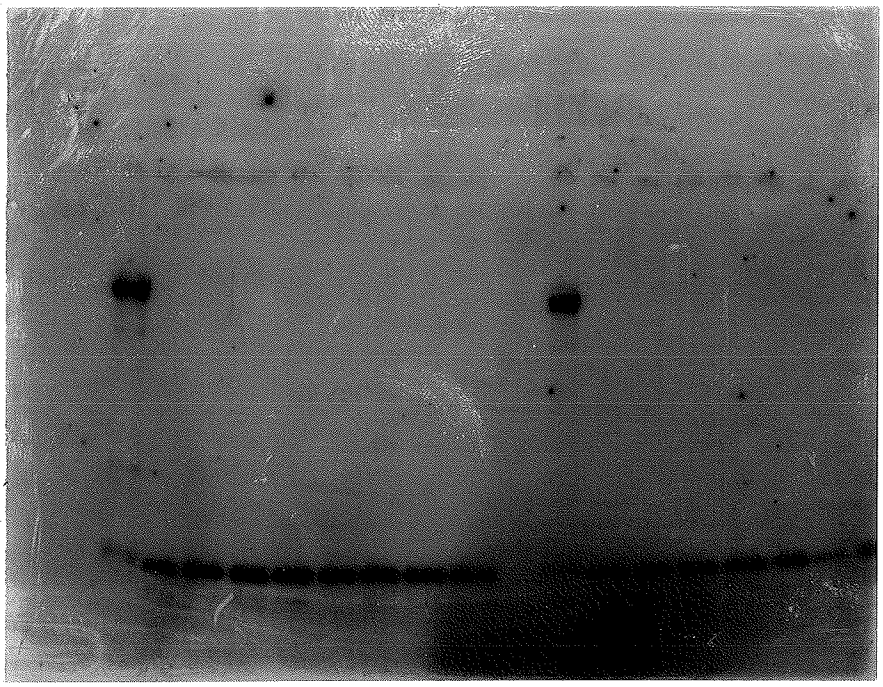
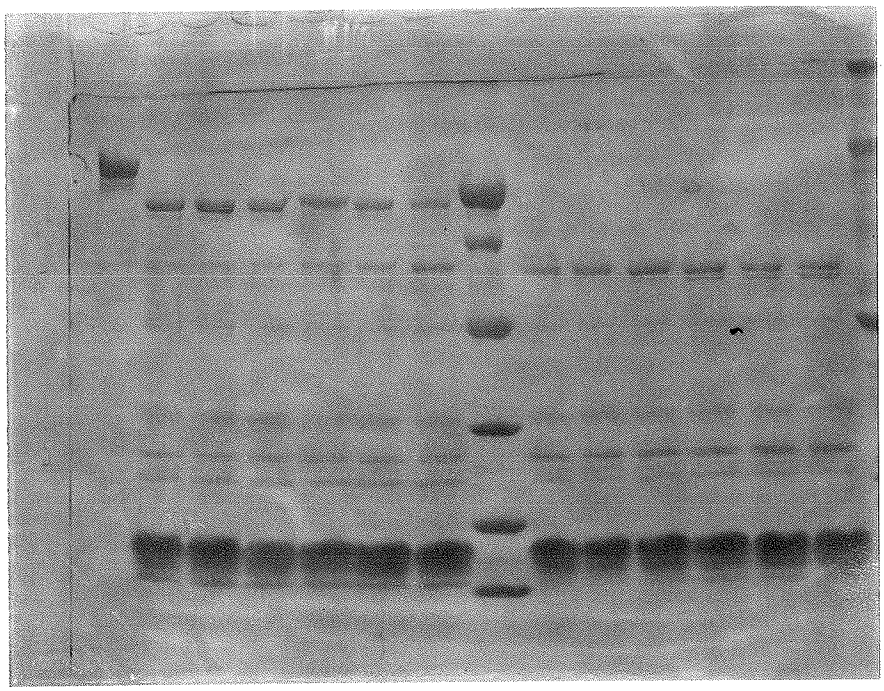


Figure 16.

An autoradiogram of a chymotryptic digest of myosin light chain kinase phosphorylated by cAMP dependent protein kinase, both with and without calmodulin bound.

Lane 1 - undigested kinase phospphorylated by protein kinase
2 - CT digest of kinase - 10 seconds
3 - CT digest of kinase - 30 seconds
4 - CT digest of kinase - 1 minute
5 - CT digest of kinase - 2 minutes
6 - CT digest of kinase - 3 minutes
7 - CT digest of kinase - 4 minutes
8 - CT digest of kinase - 5 minutes
9 - CT digest of kinase - 10 minutes
10 - undigested kinase-calmodulin complex phosphorylated by protein kinase
11 - CT digest of kinase-calmodulin compoex - 10 seconds
12 - CT digest of kinase-calmodulin complex - 30 seconds
13 - CT digest of kinase-calmodulin complex - 1 minute
14 - CT digest of kinase-calmodulin complex - 2 minutes
15 - CT digest of kinase-calmodulin complex - 3 minutes
16 - CT digest of kinase-calmodulin complex - 4 minutes

The digest was run on a 10% SDS-polyacrylamide slab gel; the gel was dried, and the autoradiogram performed.



Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 17.

A comparison of the chymotryptic and tryptic digests of myosin light chain kinase run on 7.5-20 % SDS-polyacrylamide slab gel and stained with coomassie-blue,

Lane 1 - undigested kinase
2 - CT digest - 10 seconds
3 - CT digest - 30 seconds
4 - CT digest - 1 minute
5 - CT digest - 2 minutes
6 - CT digest - 3 minutes
7 - CT digest - 5 minutes
8 - MW markers
9 - Trypsin digest - 10 seconds
10 - Trypsin digest - 30 seconds
11 - Trypsin digest - 1 minute
12 - Trypsin digest - 2 minutes
13 - Trypsin digest - 3 minutes
14 - Trypsin digest - 5 minutes.

The reaction was stopped with lima bean trypsin inhibitor.

Molecular weight markers -	Phosphorylase b	- 94,000
	Albumin	- 67,000
	Ovalbumin	- 43,000
	Carbonic anhydrase	- 30,000
	Trypsin Inhibitor	- 20,100
	Lactalbumin	- 14,000

Table 7

A Comparison of the Chymotrypsin and Trypsin Digests

Trypsin Digest	Chymotrypsin Digest	
	Kinase-Calmodulin Complex	Kinase alone
58,000	92,000	84,000
32,000	60,000	60,000
28,000*	32,000	32,000
18,000	28,000*	28,000*
15,000[2]	26,000	26,000

* Peptide which contains all of the P^{32}

[2] Peptide which bound to the calmodulin-affinity column

Phosphorylation of Chymotrypsin Digest

Introduction

In an attempt to show that the calmodulin binding domain is composed of different residues than the two phosphorylation sites, the kinase was subjected to either chymotryptic digestion followed by incubation with protein kinase, or incubation with protein kinase followed by chymotryptic digestion. It has been shown (Conti and Adelstein 1981) that in the presence of calcium and calmodulin, only one mole of phosphate is incorporated into myosin light chain kinase. There are two probable explanations for this observation. Calmodulin may actually be binding to one of the phosphorylation sites and preventing a phosphate group from being incorporated while not affecting the other phosphorylation site; or calmodulin may be binding a protein kinase recognition sequence. The second explanation involves calmodulin binding to the kinase and causing a conformational change which causes a portion of the kinase molecule to swing out and mask either a phosphorylation site or a protein kinase recognition sequence. If chymotrypsin digestion on the calmodulin-kinase complex releases a peptide which can then be phosphorylated by protein kinase at two sites, then the second explanation would appear to be correct. If, however, chymotrypsin digestion does not release a peptide which can be phosphorylated at two sites by protein kinase, then it is likely that calmodulin is remaining bound to either a phosphorylation site or a protein kinase recognition sequence. It appears that the calmodulin binding domain remains intact after both tryptic and chymotryptic digestion, as in both cases peptides containing the site will still bind to calmodulin affinity columns (Walsh et al 1982). Although it could be argued that these peptides may only contain basic resicues which are binding non-specifically to calmodulin, it has also been shown that the 95,000 peptide which is released from limited chymotryptic digestion retains its calcium sensitivity, presumably throught the action of calmodulin (Walsh et al 1982).

Methods

Phosphorylation

Myosin light chain kinase (10^{-10} M final concentration) was incubated with 10 μg/ml of the catalytic subunit of cAMP dependent protein kinase, 0.6 mM (γ-^{32}P)ATP (229 cpm/pmol), 50 mM Tris-HCl, pH 7.6, 10 mM MgAc, 10 mM βME, and either 0.2 mM EGTA or 0.2 mM $CaCl_2$ and 10 μg/ml calmodulin for 1 hour at 24°C. Phosphate incorporation was measured by removing 20 μl of the incubation mixture and spotting onto filter paper discs. The discs were washed, dried, and counted in the scintillation counter. Maximum phosphorylation of the kinase was confirmed by SDS-polyacrylamide gels, followed by an autoradiogram.

Digestion

Either 50 μl of phosphorylated myosin light chain kinase labelled with P^{32} or 50 μl of unphosphorylated kinase was incubated with chymotrypsin (1 mg/ml) at a protease:protein weight ratio of 1:500 in 20 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, at 24°C. for the digest of the kinase-calmodulin complex, $CaCl_2$ and calmodulin were added to a final free concentration of 0.5 mM. Digestion was carried out for 2 minutes. The reaction was stopped with lima bean trypsin inhibitor (1 mg/ml). Following digestion, the mixture was incubated with 10 μg/ml of the catalytic subunit of protein kinase and 0.6 mM (γ-^{32}P)ATP and either 0.5 mM calmodulin or 0.2 mM EGTA for 1 hour at 24°C. Aliquots (20 μl) of the incubation mixture were removed both before chymotrypsin digestion and after incubation with protein kinase to determine phosphate incorporation. Phosphorylation of the kinase was confirmed by SDS-polyacrylamide gel followed by an autoradiogram.

Results

It was observed that if myosin light chain kinase was incubated with calmodulin and protein kinase, 0.64 mol phosphate/mol kinase was incorporated into the kinase, whereas if the enzyme was incubated with protein kinase in an EGTA buffer, 1.45 mol phosphate/mol kinase was incorporated (Table 8 - numbers 1 and 5). This agrees with the results seen by Conti and Adelstein (1981) and indicates some form of competition between calmodulin and protein kinase.

If myosin light chain kinase which was bound to calmodulin in a kinase-calmodulin complex was treated with chymotrypsin for 2 minutes, and then calmodulin and protein kinase were both added to the digestion mixture, the amount of phosphate incorporated increased to 1.62 mol phosphate/mol kinase (Table 8 - number 4). This would indicate that after digestion of the kinase-calmodulin complex a peptide was released which protein kinase could then phosphorylate. A similar amount of phosphate (1.45 mol) was incorporated if the kinase was digested by chymotrypsin and then incubated with protein kinase alone (Table 8 - number 7). An autoradiogram was done on the fractions (Figure 18) which showed that in all instances the phosphate label was either in the intact kinase or in the 28,000 peptide shown released upon either limited tryptic or chymotryptic digestion.

If calmodulin is binding to a phosphorylation site or a protein kinase recognition sequence, then it would be expected that after digestion by chymotrypsin, the calmodulin would remain bound to the same site. If protein kinase was then added to the digest, no further phosphate should be incorporated. However, this is not the case. Following chymotrypsin digestion, a maximum amount of P^{32} could then be incorporated into the enzyme, whether or not calmodulin is present, indicating that after digestion there is no longer any competition between calmodulin and protein kinase. It is possible that calmodulin will not bind to the kinase after digestion thereby allowing protein kinase free access to the phosphorylation sites. Preliminary work by Walsh et al (1982) showing that the 95,000 peptide retains its calcium sensitivity and will bind to a calmodulin affinity column would tend to indicate

Table 8

Phosphorylation of Chymotryptic Digest of Myosin Light Chain Kinase

	Incubation Mixture[2]	Total Phosphate (pmoles)	Mol Phosphate/ Mol Kinase [3]	Standard Deviation
1)	MLCK + Calmodulin + Protein Kinase	16	0.64	0.149
2)	MLCK + Calmodulin + Protein Kinase / + CT	15.6	0.65	0.092
3)	MLCK + Calmodulin / + CT / + Protein Kinase	27.02	1.42	0.055
4)	MLCK / + CT / + Calmodulin + Protein Kinase	29.31	1.62	0.098
5)	MLCK + EGTA + Protein Kinase	23.5	1.45	0.08
6)	MLCK + EGTA + Protein Kinase / + CT	24.4	1.46	0.09
7)	MLCK + EGTA / + CT / + Protein Kinase	26.8	1.45	0.086

[2] MLCK, calmodulin, and protein kinase were incubated for 1 hour at 24°C, either before the digest or after the digest. CT was allowed to digest for 2 minutes before the reaction was stopped. / indicates the addition of further reactants.

[3] Calculated as a means of 9 results.

NOTICE/AVIS

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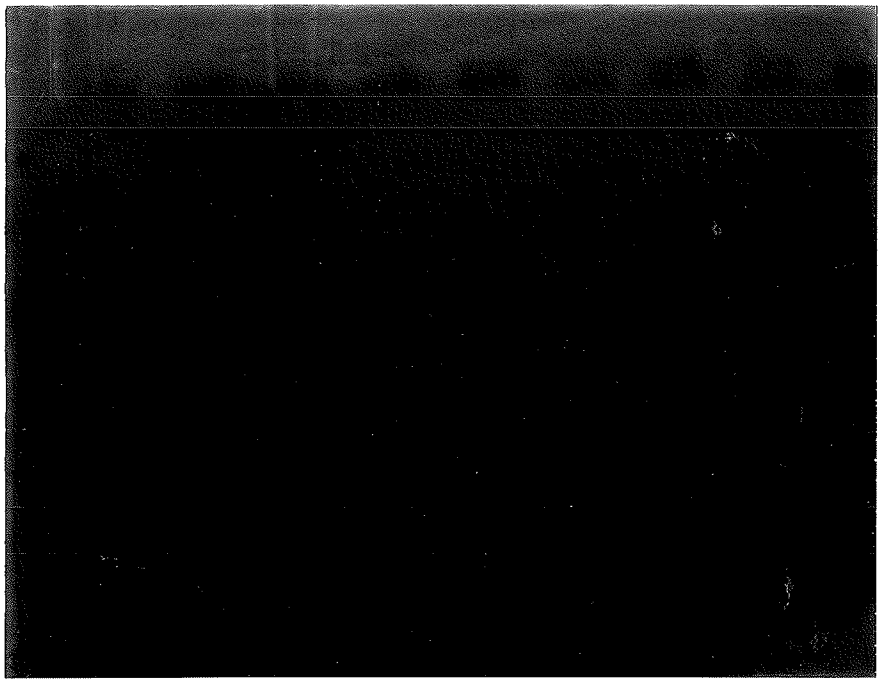

-- 130,000

-- 28,000

Lane 1 2 3 4 5 6 7

Figure 18.

An autoradiogram done on a 7.5-20% SDS-polyacrylamide gel run on the combined chymotryptic digest and protein kinase assay (Table 7).

Lane 1 - undigested MLCK + calmodulin + protein kinase
2 - MLCK + calmodulin + protein kinase digested for 2 minutes with CT
3 - MLCK + calmodulin digested for 2 minutes with CT, with addition of PK
4 - MLCK digested for 2 minutes with CT, followed by addition of both protein kinase and calmodulin
5 - MLCK digested with CT for 10 seconds
6 - MLCK digested with CT for 2 minutes
7 - MLCK digested with CT for 2 minutes followed by the addition of protein kinase.

that the calmodulin binding site remains intact after digestion, but further work will have to be done to conclusively show this. The results obtained here would tend to indicate that calmodulin is not binding to either a phosphorylation site or a protein kinase recognition sequence.

Discussion

In trying to understand any form of competition between two proteins binding to the same substrate, both direct interference due to the involvement of the same residues and indirect interference caused by conformational changes induced by one protein binding resulting in an interference with the second protein binding must be taken into account. In dealing with enzymes, a recognition sequence which the enzyme binds to may be present on the protein substrate and be separable and distinct from the catalytic site.

It has been shown clearly by several researchers that calmodulin binding to myosin light chain kinase causes a conformational change. This has been confirmed in the chymotryptic digest presented here (p. 57). There are several ways in which calmodulin binding to the kinase could affect the phosphorylation mechanism of protein kinase. One involves the binding of a part of the calmodulin molecule directly to one of the phosphorylation sites with both the phosphorylation site and the calmodulin binding site containing the same residues. Preliminary work by Walsh et al (1979) and the evidence presented in this thesis indicate this not to be the case. After a trypsin digest, a peptide which contains both phosphorylation sites can be separated from a peptide which binds to a calmodulin affinity column (p. 47). This would indicate that different residues are involved in each site. However, it has not yet been conclusively shown that the peptides which bound to the column actually contain the calmodulin binding domain rather than a predominance of basic residues.

Another mechanism of competition involves an indirect effect of calmodulin binding. Either a part of the calmodulin molecule itself that is not bound to the kinase physically restricts protein kinase access ro the phosphorylation sites, or the conformational change induced by calmodulin binding results in one of the phosphorylation sites becoming hidden from protein kinase. There has been no evidence presented to date which distinguishes these two forms of competition, and either mechanism may be involved in the calmodulin-protein kinase competition for myosin light chain kinase.

A similiar relationship to the above mechanism exists between calmodulin binding and protein kinase recognition sequences. Because protein

kinase is an enzyme, there exists the possibility that myosin light chain kinase contains a recognition sequence for the enzyme as well as a catalytic site consisting of two phosphorylation sites. The calmodulin binding domain may consist of the same residues involved in the protein kinase recognition sequence. However, this does not appear to be the case. Evidence presented here indicates that after chymotrypsin digestion, protein kinase is able to fully phosphorylate a 28,000 dalton peptide whether or not calmodulin is present (p. 62). This ability to phosphorylate a peptide by protein kinase indicates the presence of a functional recognition sequence as well as functional phosphorylation sites. The lack of any competition between calmodulin and protein kinase in a chymotryptic digest of myosin light chain kinase would indicate that the same residues are not involved in both the calmodulin binding domain and the phosphorylation sites or protein kinase recognition sequence. However, if calmodulin binding is causing a conformational change which is restricting access of protein kinase to the recognition sequence these same results would be seen. Further work will have to be done to try and differeniate between these forms of competition with respect to myosin light chain kinase and calmodulin - protein kinase.

Conclusion

Work done by Conti and Adelstein (1980; 1981) and Walsh et al (1979; 1980; 1982; 1983) has indicated the presence of at least three domains in myosin light chain kinase: an active site, a calmodulin binding domain; and two phosphorylation sites. As isolation procedures for the enzyme become more refined, the molecular weight of the isolated kinase has increased to a generally seen protein of 130,000 daltons. However, with the isolation of a 155,000 dalton protein (Walsh et al 1982) and a 141,000 dalton and 136,000 dalton kinase (Walsh et al 1982), it may be that the 130,000 kinase is a proteolytic product of a larger protein.

The mechanism for control of smooth muscle contraction as proposed by Adelstein and Klee (1981) involves a dual regulation of myosin light chain kinase by calmodulin and protein kinase. The kinase is activated by calmodulin binding and will phosphorylate myosin light chains, resulting in a form of myosin which will interact with actin. Protein kinase, in turn, will phosphorylate myosin light chain kinase, which results in a decrease in the calcium sensitive threshold for contraction in smooth muscle. This decrease in calcium sensitivity can be overcome by increasing the level of calmodulin in the system (Kerrick and Hoar 1981). This relationship between calmodulin binding and phosphorylation by protein kinase has been seen in other proteins besides myosin light chain kinase, leading researchers to investigate this observation (Malencik and Anderson 1982; Malencik et al 1982).

Several ideas have been put forward to explain this phenomenon. These include the binding of calmodulin to one of the phosphorylation sites; the binding of calmodulin to protein kinase recognition sequence (Malencik and Anderson 1982); or a conformational change induced by calmodulin in one area of the kinase affecting the area phosphorylated by protein kinase (Walsh et al 1982). To see if the different domains can be separated, proteolytic studies have been employed to try and resolve the relationship between protein kinase and calmodulin. Walsh et al (1982) have shown that limited chymotryptic digestion on the kinase alone will result in a 95,000 dalton protein which will bind to a calmodulin affinity column. This 95,000 dalton protein also retains its calcium sensitivity, indicating

the calmodulin binding domain is intact. However, no phosphate can be incorporated into the peptide. Walsh et al (1979) also used trypsin to show that a peptide containing the phosphorylation sites could be separated from a peptide which bound to a calmodulin affinity column.

Experiments reported in this thesis have confirmed Walsh's findings, and have also shown that the competition between calmodulin and protein kinase is abolished in a chymotrypsin digest of myosin light chain kinase. If calmodulin and protein kinase were competing for the same site, then digestion of the kinase should not affect the competition between the two. However, as the competition is abolished in the digest, it indicates the calmodulin binding site and the phosphorylation sites contain different residues. No other forms of competition may be differentiated by these results, and much more work will have to be done in this area to fully explain the competition between calmodulin and protein kinase.

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