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A COMPARISON OF THE PROPERTIES OF  
RABBIT SKELETAL  $\alpha$  AND  $\beta$ -TROPOMYOSINS

by



ELKE MONIKA LOHMEIER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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DEDICATION

To Hans and to our respective families

## ABSTRACT

The two major forms of rabbit skeletal tropomyosin (designated  $\alpha$  and  $\beta$ ) have been separated on a CM-cellulose column in 0.8 M urea, 50 mM Na formate pH 4.0, 1 mM EDTA and 5 mM DTT. Following renaturation,  $\alpha$ -Tm ( $\alpha,\alpha$ -homodimer) and  $\beta$ -Tm ( $\beta,\beta$ -homodimer) were characterized with respect to their abilities to interact with the other members of the thin filament.

The technique of viscosity demonstrated no differences between the two forms of tropomyosin to polymerize in a head to tail fashion. As well, no alteration in the ability of whole troponin to affect these end to end contacts was demonstrated by viscometry. However, definite differences between  $\alpha$  and  $\beta$ -tropomyosins with respect to their troponin binding capacities were seen when they were passed through a troponin Sepharose 4B affinity column.  $\beta$ -Tm eluted earlier (0.12 M KCl) than  $\alpha$ -Tm (0.2 M KCl), suggesting that the  $\beta$ -form has a weaker ability to associate with the troponin complex. These results were substantiated with gel filtration studies using the cyanogen bromide fragment CBI of Tn-T. Again, the  $\beta$ -tropomyosin had the weaker interaction of the two Tm forms.

The association between radioactively labelled  $\alpha$  and  $\beta$ -tropomyosins and actin filaments was studied with the technique of co-sedimentation. A stronger affinity with F-actin at the higher ionic strengths was demonstrated for  $\beta$ -Tm. When the magnesium ion concentration was varied, the actin binding profiles for both forms of Tm were similar.

With this background knowledge, the  $\alpha$  and  $\beta$ -tropomyosins were analysed with respect to their behaviour as regulatory proteins in a reconstituted muscle system (actin, troponin, tropomyosin and myosin S-1), both



in the presence and absence of calcium. Studies at three different S-1 to actin concentrations (1 to 7, 1 to 2 and 2 to 1) demonstrated that  $\beta$ -Tm was less able to potentiate the acto-S-1 ATPase activity. Investigations involving the use of non-polymerizable tropomyosin showed that potentiation is largely dependent on head to tail overlap. Thus  $\beta$ -Tm's weaker association with troponin may affect its ability to function cooperatively in the reconstituted acto-S-1 ATPase system.

These results suggest that  $\beta$ -Tm may be slightly more of a "structural" and less of a "regulatory" protein relative to  $\alpha$ -Tm. Thus the higher  $\beta$ -content in embryonic muscles may assist in the organization and stabilization of the newly forming thin filaments.

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I would first like to thank my supervisor, Dr. L.B. Smillie for suggesting a suitable masters project to me, and for his integrity and guidance in the course of my studies.

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## ABBREVIATIONS

A	-	Actin
ADP	-	Adenosine-5'-diphosphate
ADP·Pi	-	Adenosine-5'-diphosphate with non-covalently bound phosphate
Ala	-	Alanine
Asn	-	Asparagine
Asp	-	Aspartic acid
ATP	-	Adenosine-5'-triphosphate
A $\lambda$	-	Absorbance of a protein at the given wavelength $\lambda$
A1	-	Alkali light chain 1 of myosin
A2	-	Alkali light chain 2 of myosin
$\alpha$ -Tm	-	Tropomyosin composed of two $\alpha$ subunits ( $\alpha,\alpha$ -homodimers)
$\beta$ -MeOH	-	Mercaptoethanol
$\beta$ -Tm	-	Tropomyosin composed of two $\beta$ subunits ( $\beta,\beta$ -homodimers)
CB1	-	Residues 1 to 151 of Tn-T
CB2	-	Residues 71 to 151 of Tn-T
CM	-	Carboxymethyl
CNBr	-	Cyanogen bromide
COOH-terminal	-	Carboxyl terminal
C-Tm	-	Cardiac tropomyosin
Cys	-	Cysteine
DEAE	-	Diethyl amino ethyl
DFP	-	Diisopropyl fluoro phosphate
DTNB	-	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	-	Dithiothreitol
d <sup>3</sup> H <sub>2</sub> O	-	doubly distilled, deionized water



- d - Diameter
- EDTA - Ethylenediamine tetraacetic acid
- EGTA - Ethyleneglycol-bis-( $\beta$ -amino ethyl ether) N,N'-tetraacetic acid
- $E_{\lambda}^{1\% \text{ cm}^{-1}}$  - Absorbance of a 1% protein solution in a 1 cm pathlength at wavelength  $\lambda$
- F-actin - Filamentous actin
- g - Grams
- G-actin - Globular actin monomers
- G-S - Guba Straub
- h - height
- His - Histidine
- HMM - Heavy meromyosin
- HMM S-1 - Heavy meromyosin subfragment-1, the myosin head, or S-1
- Ile - Isoleucine
- Leu - Leucine
- M - Myosin
- Met - Methionine
- NH<sub>2</sub>-terminal - Amino terminal
- NPTm - Non-polymerizable tropomyosin
- Tm - Tropomyosin
- Tn - Troponin
- Tn-C - Calcium binding subunit of troponin
- Tn-I - Inhibitory subunit of troponin
- Tn-T - Tropomyosin binding subunit of troponin
- Tris - Tris-(hydroxymethyl) amino methane
- T<sub>1</sub> - Residues 1 to 158 of Tn-T
- T<sub>2</sub> - Residues 159-258 of Tn-T

S-1 - Myosin head (HMM S-1)

s<sup>-1</sup> - Turnover number; molecules substrate per molecule enzyme per second

SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Val - Valine

v - Volume

$\lambda$  - Wavelength

## CHAPTER 1

### INTRODUCTION

The study of muscle is the study of movement, a fundamental process of life. The conversion of the chemical energy stored in ATP into mechanical energy has provided fertile research material for decades. This makes it extremely difficult to do justice to the area in this introduction. The following review articles can be referred to when a more indepth understanding is required; Adelstein and Eisenberg (1980), McCubbin and Kay (1980), Taylor (1979), Mannherz and Goody (1976), Squire (1975) and Weber and Murray (1973).

Movement in vertebrates is accomplished by specialized structures called muscles. These fall into three categories:

- 1) Cardiac or heart muscle
- 2) Smooth muscle
- 3) Skeletal muscle.

The first two categories are under involuntary and hormonal control whereas the third category is under voluntary control. For the sake of brevity, further discussion will be limited to the skeletal muscle system.

#### A. SKELETAL MUSCLE MORPHOLOGY

Skeletal muscle is composed of bundles of individual muscle fibres. Each fibre is actually a single multinucleated cell. It is about 100  $\mu\text{m}$  in diameter but can attain lengths of 30 cm or more. The fibres are surrounded by plasma membranes called sarcolemmas which are in close association with nerves. Dissection of a muscle fibre (cell) reveals the myofibrils. These thin cylindrical units run the entire length of the cell

and are about 10  $\mu\text{m}$  in diameter. Myofibrils are surrounded by a specialized membrane system which is called the sarcoplasmic reticulum. A system of transverse tubules provides the communication link between the sarcolemma and the sarcoplasmic reticulum.

Closer inspection of the myofibril interior under a phase contrast microscope shows the characteristic banded appearance which accounts for the name "striated" muscle. The bands arise due to repeating units of two distinct types of smaller filaments. Each repeating unit (see Fig 1) is called a sarcomere.

The thick filaments are composed mainly of myosin. They form the bulk of the dark central portion of the sarcomere known as the A band. A lighter H zone in the middle of this band is bisected by the M line, which links neighbouring sets of bipolar thick filaments together.

The thin filaments are composed of three types of proteins; actin, troponin and tropomyosin in a 7:1:1 molar ratio. These composite filaments are attached to the Z lines at the boundaries of the sarcomere. They overlap with the thick filaments in the A band.

Taken in cross section it can be seen that in the overlap region each thick filament is surrounded by six hexagonally arrayed thin filaments whereas each thin filament is surrounded by three/triangularly arranged thick filaments. Under certain conditions electron microscopy has shown that short projections emanate from the thick filaments to the thin filaments. They have been called crossbridges.

Before a deeper understanding of muscle can be reached, the individual proteins making up these filaments must be discussed. All molecular weights listed below pertain to the rabbit skeletal system.

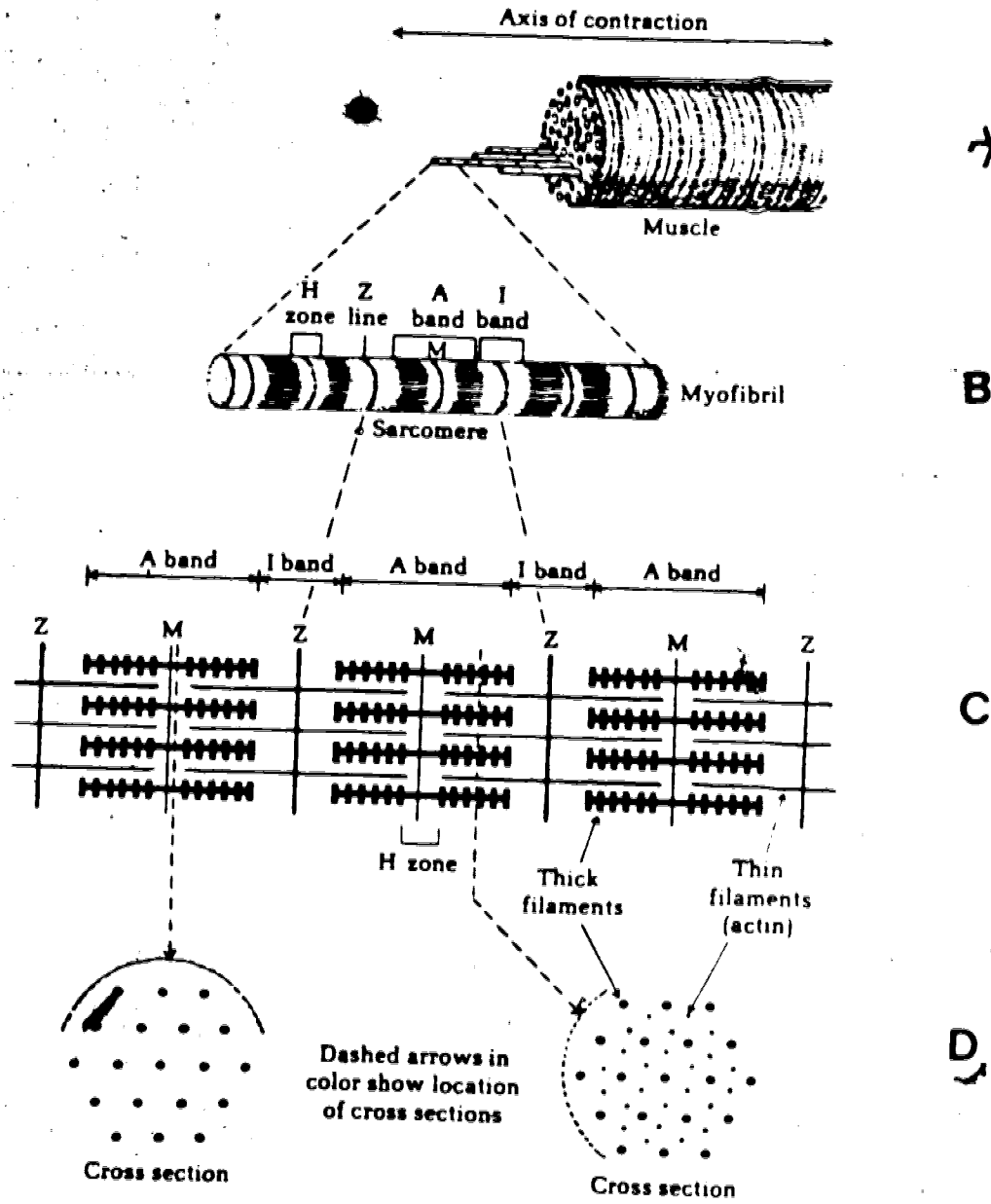


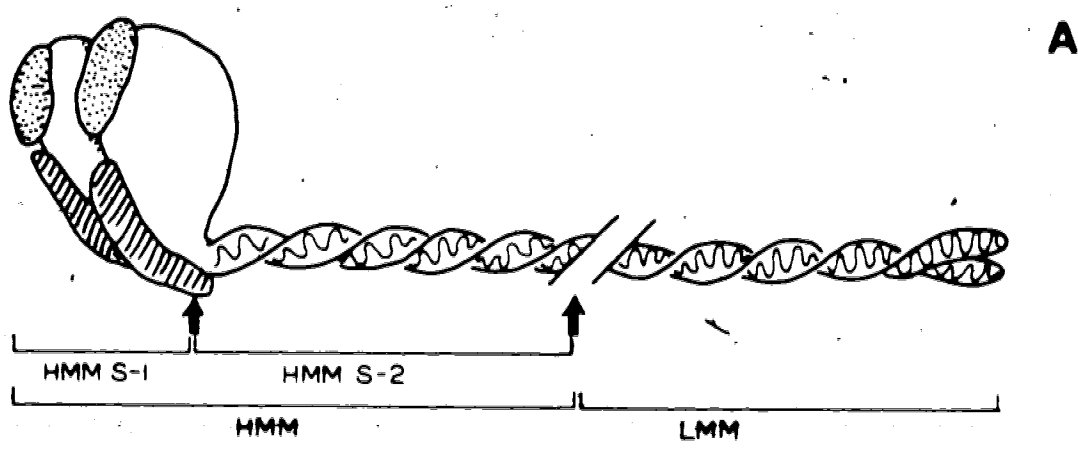
Fig. 1. Diagrammatic representation of the structure of striated muscle.  
 a) A muscle fibre (cell) in cross section showing the myofibrils.  
 b) Enlargement of one myofibril showing the repeating sarcomeres.  
 c) Schematic illustration representing the thick and thin filaments of several sarcomeres.  
 d) Cross sections of sarcomeres illustrating the relationship between the thick and thin filaments in the myofibril.  
 (From Lehninger, 1975).

## B. MYOSIN AND THE THICK FILAMENT

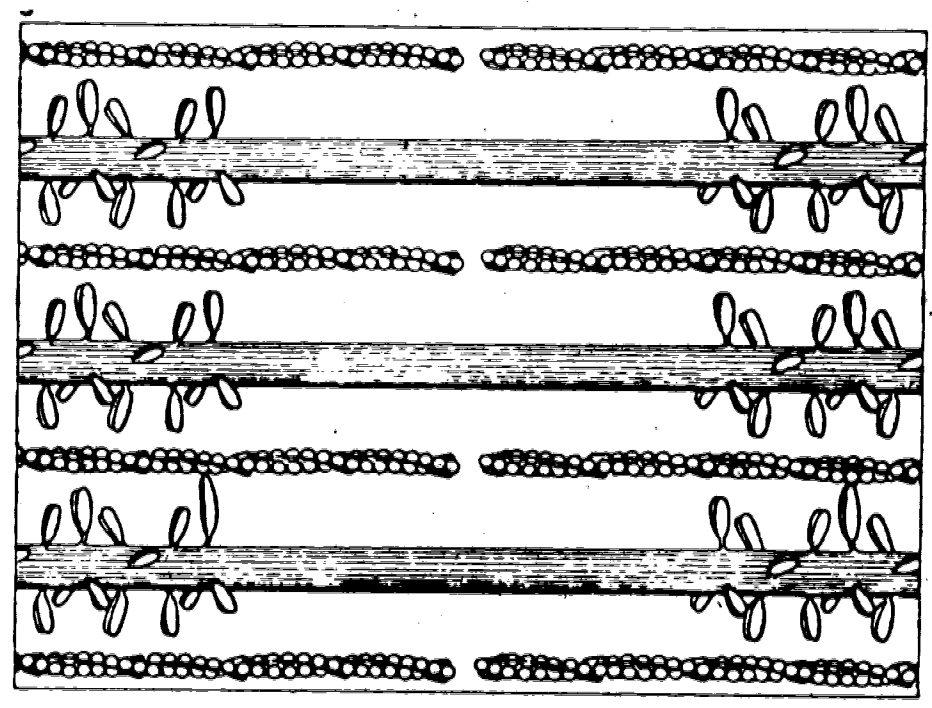
Fig. 2a schematically represents one myosin molecule and Fig. 2b illustrates its arrangement in the thick filaments. Myosin is highly asymmetrical. It is made up of six polypeptide chains, two major ones (heavy chains) and four minor ones (light chains). The heavy chains have a molecular weight of 200,000 each. They coil about one another with their highly  $\alpha$ -helical carboxyl terminal regions. The amino terminals are more globular in shape. They form the "heads" or the "crossbridges" since they associate with the actin monomers in the thin filaments. These heads have the ability to cleave ATP.

The myosin molecule is not complete without its light chains. These fall into two categories, essential and nonessential. Each of the globular heads binds one member of these two classes. The nonessential light chains are not required for ATPase activity. They are known as the DTNB light chains since they can be largely removed from their association with the heavy chains by treatment with 5,5'-dithiobis-(2-nitrobenzoic acid). The essential light chains, on the other hand, are required for activity. They can only be removed with much harsher treatments such as alkali; thus they are often referred to as the alkali light chains. Two variations of this class of light chain occur. The A-1 light chain has a molecular weight of 21,000 whereas the A-2 light chain is smaller, having a molecular weight of only 16,500.

In high salt myosin is monomeric; however at more physiological ionic strengths (0.15 M KCl) bipolar aggregates resembling thick filaments form spontaneously. The rod portions of the heavy chains form the core of the filament. The globular heads protrude from this core and are arranged helically around it.



A



B

Fig. 2a. A diagrammatic representation of a single myosin molecule consisting of 2 heavy chains, 2 alkali light chains (stippled) and 2 DTNB light chains (hatched). Points for enzymatic cleavage are indicated by heavy arrows. The myosin fragments HMM (heavy meromyosin and its subfragments S-1 and S-2) and LMM (light meromyosin) are bracketed below.

Fig. 2b. Model for the arrangement of the myosin molecules in the thick filament illustrating their bipolar arrangement. (From Cohen, 1975).

Myosin can be cleaved at several points (as indicated by heavy arrows in Fig. 2a) to yield single-headed S-1 (HMM S-1) or double-headed HMM (heavy meromyosin) fragments. Both of these are soluble at lower salt concentrations and both retain the ability to cleave ATP (Lowey et al., 1969).

### C. THE THIN FILAMENT PROTEINS

The thin filament is composed of three proteins; actin, troponin and tropomyosin. Each will be briefly discussed in this section.

Actin is a globular protein with a molecular weight of about 42,000. For a review of its properties, see Oosawa and Kasai (1971). Each actin molecule has one bound nucleotide and one bound divalent cation. Both are necessary for stability of the polypeptide chain. Monomeric, globular G-actin will spontaneously aggregate into filamentous F-actin if either the ionic strength and/or the concentration exceed certain critical levels. The ATP is hydrolysed into ADP and inorganic phosphate during the polymerization process. In skeletal muscle all the actin is in the filamentous form. The filaments are composed of two strands of F-actin twisted helically about one another (Fig. 3). Actin has been sequenced by Collins and Elzinga (1975) and is found to be highly conserved in the different cells and tissues from which it is isolated (Vandekerckhove and Weber, 1978).

Troponin is actually a complex of three polypeptide chains, each with a special function. The largest subunit is Tn-T, so called because it binds to tropomyosin. It has been sequenced by Pearlstone et al. (1976), has a molecular weight of 30503 and is basic.

Tn-I is also basic. It has a molecular weight of 21,000 and forms the inhibitory component of the troponin complex because it is found to be



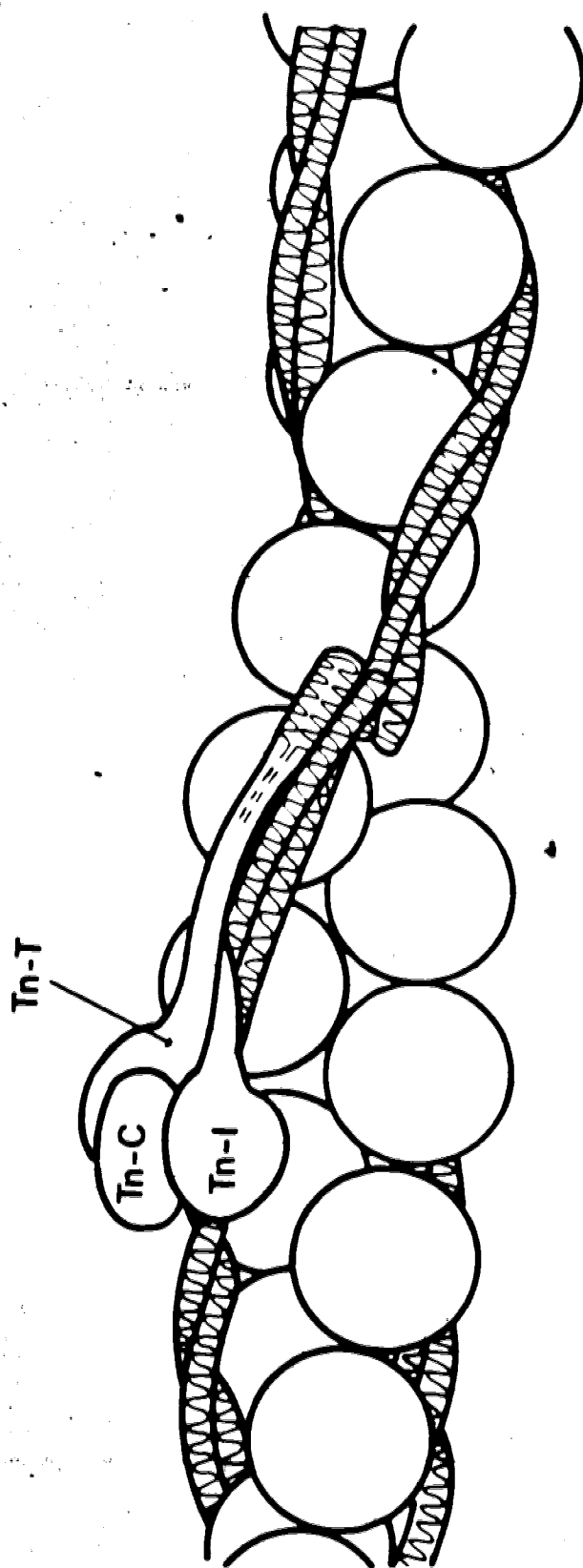


Fig. 3 A model for the molecular arrangement of the thin filament proteins. Two strands of actin molecules (open circles) form the core of the structure. Tropomyosin molecules are illustrated as coiled-collars which lie in both grooves of the actin filament. The troponin complex (Tn-I, Tn-C and Tn-T) interacts with the terminal 1/3 of the tropomyosin molecule. Each tropomyosin spans 7 actin molecules and is associated with one troponin complex. This forms the basic regulatory unit of the thin filament. (Adapted from Cohen, 1975).

capable of inhibiting the actomyosin ATPase in conjunction with tropomyosin. The primary sequence has been derived by Wilkinson and Grand (1975).

Tn-C is the smallest subunit and has a molecular weight of 17840. It is an acidic protein and is thus capable of interacting strongly with the other basic subunits. Tn-C has four calcium binding sites, two of which function in the physiological range of calcium concentration. When  $Ca^{2+}$  is bound, Tn-C undergoes distinct conformational changes (McCubbin and Kay, 1980). Tn-C alone can reverse the Tn-I inhibition of the actomyosin ATPase. The sequence has been determined by Collins (1974).

Tropomyosin is the protein which links actin and troponin together. It is a long (41 nm) rod-like protein which lies in the grooves formed from the two actin filaments. One tropomyosin molecule spans approximately seven actin monomers and binds one troponin complex, thus forming the basic unit of the thin filament (see Fig. 3).

Tropomyosin is formed from two 33,000 dalton subunits which are highly  $\alpha$ -helical and wrap around each other as coiled-coils (for a review see Smillie, 1979) to form a molecule with molecular weight 66,000. Tropomyosin overlaps in a head to tail fashion, thus providing a cooperative link through the thin filament. Tropomyosin will be discussed in greater detail later in the thesis.

#### D. MUSCLE CONTRACTION AND ITS REGULATION

Muscle contraction occurs first with a signal to the nerve controlling a group of muscle fibres (motor unit). The nerve impulse depolarizes the sarcolemma which in turn transmits the message via the transverse tubules to the sarcoplasmic reticulum. Calcium stored within the

this membranous system now enters the myofibrils where it is bound by troponin C. The troponin complex undergoes a conformational change which is transmitted via tropomyosin to the F-actin monomers. Actin is now able to interact favourably with the myosin heads in a cyclic manner (see Fig. 4). The energy released from the ATP which is hydrolysed during each cycle is used to move the two sets of filaments past one another. Thus the overall effect is to shorten the sarcomere length but not to alter the lengths of the filaments themselves. These concepts led to the proposal of the sliding filament model of muscle contraction (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954).

Relaxation occurs in the reverse manner. After the nerve impulse stops, calcium is actively transported back into the sarcoplasmic reticulum and its concentration reaches the resting state of  $10^{-7}$  M. The regulatory proteins alter their conformations back to the original inhibitory states and the favourable actin-myosin interaction ceases.

The question now arises as to how troponin and tropomyosin are able to exert their influence. X-ray diffraction analysis and actin paracrystal studies show the tropomyosin filament moving closer into the F-actin groove when calcium levels are high (active state). This movement would possibly reveal the myosin binding sites of the actin monomers, and would allow the two proteins to interact. Fig. 5 illustrates the basic concepts of the steric blocking model of regulation (Haselgrove, 1972; Huxley, 1972; Wakabayashi *et al.*, 1975; Parry and Squire, 1973). Although this model has been a useful working hypothesis for a number of years, recent observations have indicated that the regulatory process may operate at least in part through an allosteric mechanism possibly involving a conformational change in the F-actin monomers (see chapter 4 for further discussion).

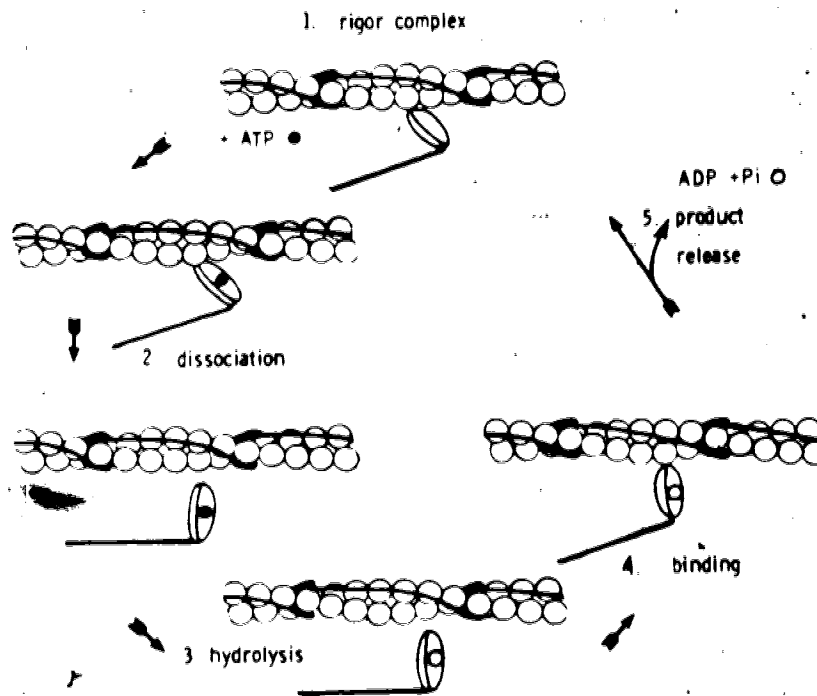


Fig. 4. Model for the Actomyosin ATPase Cycle.

- 1) Nucleotide-free myosin binds strongly as a rigor complex to F-actin. It has a  $45^\circ$  binding angle relative to the thin filament.
- 2) The binding of ATP to myosin weakens the interaction with actin and results in dissociation. The myosin reverts back to a  $90^\circ$  state (binding angle).
- 3) Myosin hydrolyses the ATP to ADP·Pi. It now regains its high affinity for actin.
- 4) Binding to actin facilitates the release of products.
- 5) As product release occurs, the myosin head goes from the  $90^\circ$  state to the  $45^\circ$  state. This change in angle represents the **POWER STROKE**.

Many repetitions of this basic cycle result in a movement of the two sets of filaments past one another.

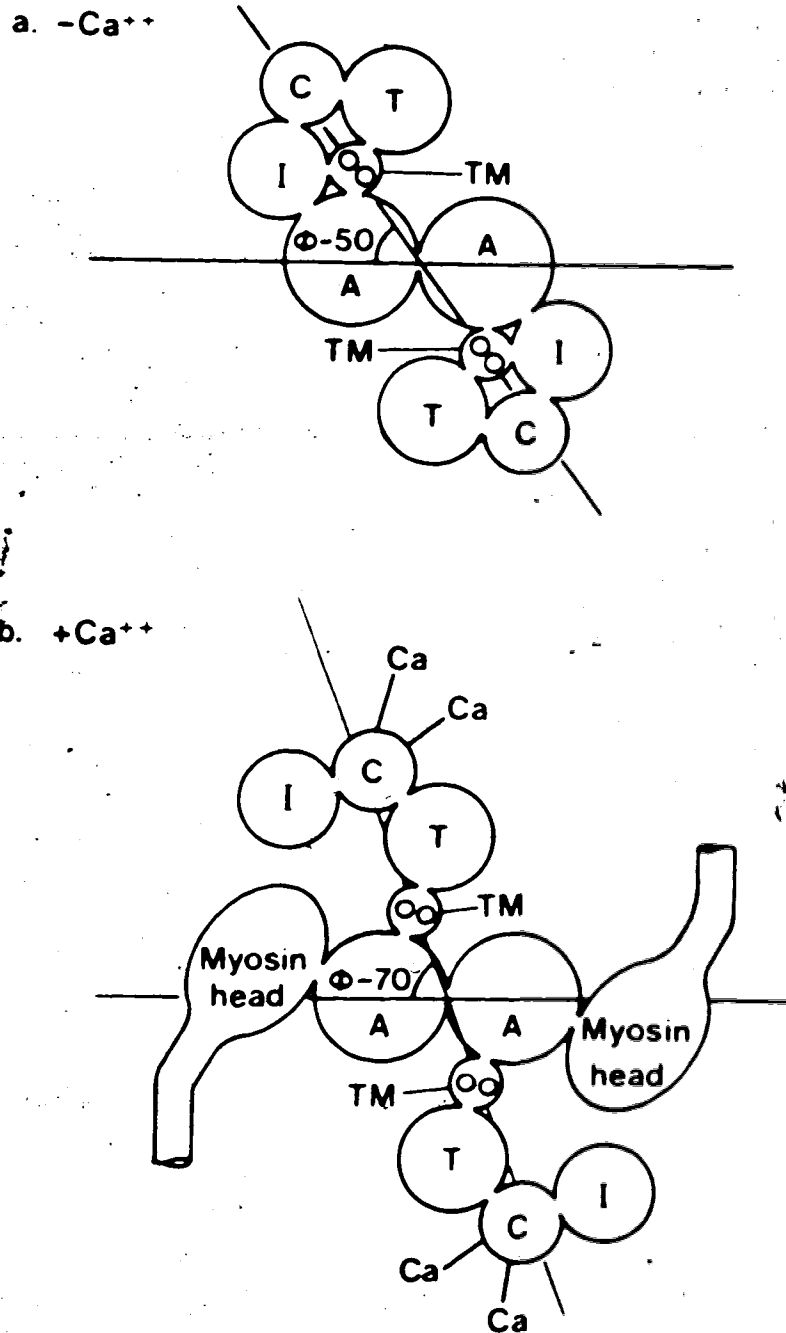


Fig. 5. The Steric Blocking Model (the thin filament is in cross section).

- In the absence of calcium, Tn-I binds to actin and anchors the tropomyosin to the periphery of the actin grooves. In this position, tropomyosin is postulated to physically block the binding of the myosin heads.
- In the presence of calcium, a conformational change in the troponin complex is transmitted to tropomyosin resulting in a movement closer into the F-actin grooves which opens up the myosin binding sites.

(From McCubbin and Kay, 1980).

## E. TROPOMYOSIN IN DETAIL

The purpose of this section of the introduction is to go into greater detail on tropomyosin (Tm), as the bulk of the work in this thesis is concerned with its nature. Further details will be supplied at the beginning of each subsequent chapter.

Tropomyosin was first purified and crystallized by Bailey (1948) but its function as a regulatory protein was not elucidated until later (Ebashi and Kodama, 1966). Physicochemical studies have shown it to be rod-like in shape with greater than 95%  $\alpha$ -helical content (Cohen and Szent-Gyorgi, 1957), which makes it the smallest member of the  $\alpha$ -fibrous group of proteins. Crick (1953) postulated that  $\alpha$ -fibrous proteins would have a coiled-coil structure in which two or three right-handed  $\alpha$ -helices twisted around each other to form rope-like strands. He also predicted that the sequence of these proteins would have to have regular repeats of non-polar amino acids every 3.5 residues in order for the coils to interact hydrophobically as they twisted about one another. It was to be another twenty years before this hypothesis could be validated.

In the meantime solution studies on tropomyosin showed that it had a monomer molecular weight of 66,000 in high salt, but only 33,000 when reduced and denatured (Woods, 1967). In low salt the molecules had the ability to polymerize end to end, giving a characteristic increase in viscosity (Kay and Bailey, 1960).

Caspar et al. (1969) investigated the ability of Tm to form a variety of ordered aggregates under appropriate isoelectric conditions. True crystals are mostly composed of water, but have provided an estimate of the molecular length of the molecule ( $410 \pm 4 \text{ \AA}$ ). Tropomyosin paracrystals are formed in the presence of divalent cations and provide useful in-

formation when they are stained with uranyl salts. Under the electron microscope these aggregates show detailed banding patterns (Stewart and McLachlan, 1976) with a periodicity of 395 Å.

The complete sequence of  $\alpha$ -Tm (Stone et al., 1974) has been analysed in detail by a number of researchers. The 284 residue polypeptide chain is indeed composed of regular alternating patterns of polar and non-polar amino acids, confirming Crick's earlier predictions. When the sequence is divided into groups of seven amino acids each, it is shown that in each heptapeptide there are two series of non-polar amino acids (series I and II), one every 3-4 residues (Stone et al., 1974). These hydrophobic residues form the core of the coiled-coil. McLachlan and Stewart (1975) illustrate this nicely (Fig. 6). Each of the seven amino acids in one "period" is labelled alphabetically from a to g. Residues a and d are generally found to be non-polar (they correspond to series I and II above) and they interact hydrophobically with residues d' and a' from the opposite coil. The sequence indicates that for the most part, residues in position g are basic and those in position e are acidic. This also provides evidence that the two tropomyosin subunits are running in the same direction, since the opposite arrangement would lead to repulsion due to the juxtapositioning of the similar charges. Since the unique Cys in position 190 can become oxidized without altering the molecular weight of Tm (66,000), the polypeptide chains are considered to be unstaggered (Johnson and Smillie, 1975; Lehrer, 1975). Given that each amino acid in such a coiled-coil has a mean residue of translation of 1.49 Å, the calculated length of the Tm molecule is equal to  $284 \times 1.49$ , or 423 Å. This is slightly longer than the X-ray crystallographic estimate of  $410 \pm 4$  Å. The only way both values can be correct is if the

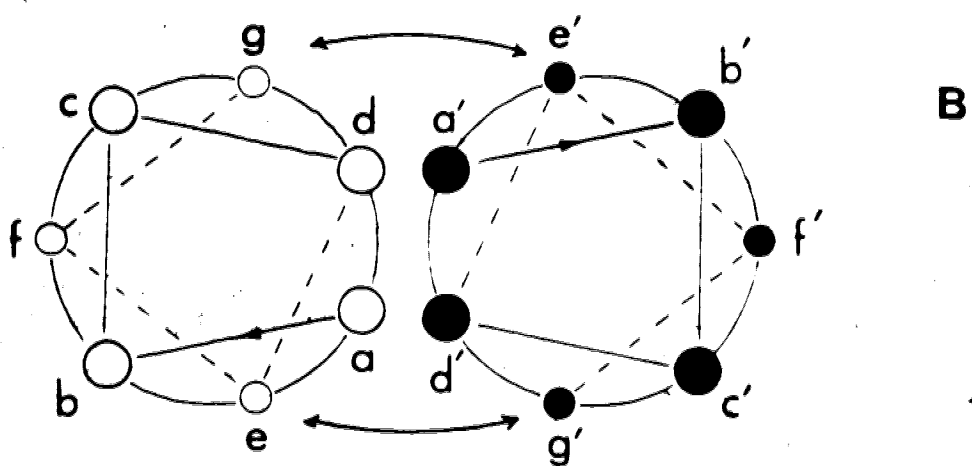
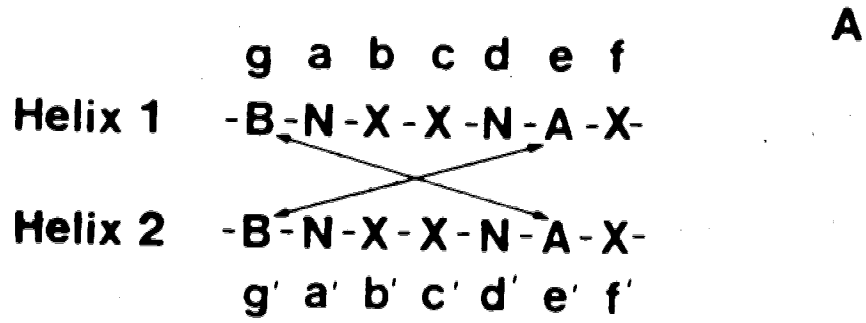


Fig. 6a. One period is illustrated for each of 2 tropomyosin subunits (the amino acids are indicated in capital letters). The arrows indicate the ionic interactions between g-e' and e-g' amino acids.

Fig. 6b. A cross section of the two tropomyosin chains illustrating an end on view (from the COOH-terminal) for a distance of one period. The helices interact hydrophobically (d-a' and a-d') and electrostatically (as indicated by the two-headed arrows). (From Smillie, 1979).



tropomyosin molecules overlapped by 8 or 9 residues. This indeed has been shown to be the case, since enzymatic removal of several COOH-terminal amino acids results in a "non-polymerizable tropomyosin" (Mak and Smillie, 1981a). Head to tail overlap also allows the periodic features of the sequence to be carried over from one molecule to another.

The advent of polyacrylamide gel electrophoresis as well as amino acid sequence analysis showed that preparations of tropomyosin are not always homogeneous. Cummins and Perry (1973) separated two major forms of rabbit skeletal Tm (designated  $\alpha$  and  $\beta$ ) of a CM-32 cellulose column in 8 M urea, and showed them to be distinct polymorphic entities (see the next section for details).

The sequence of  $\beta$ -Tm (Mak et al., 1979) differs from that of  $\alpha$ -Tm by 39 residues out of a total of 284. Only two substitutions lead to charge differences (net negative); the rest are chemically similar (Fig. 7). Most of the replacements in  $\beta$ -Tm occur in positions c and f, which are less critical for the stabilization of the coiled-coil, but are more important for the interaction with other molecules.

Once the details of the Tm structure were worked out, it became interesting to try and predict how this protein would interact with the other members of the thin filament, namely actin and troponin.

#### a) The Tropomyosin-Actin Interaction

The binding of tropomyosin to F-actin had been demonstrated by viscosity, ultracentrifugation, flow birefringence and optical rotatory dispersion methods (Drabikowski and Nowak, 1968; Tanaka, 1972). That Tm moves 10 to 15 Å relative to actin during the contractile process is also well documented (Haselgrove, 1972; Huxley, 1972; Parry and Squire, 1973). This movement led to the proposal of the steric blocking model

C Th	5	10	15	20	25	30	35
S B-Th	Act-Asp-Ala-Ile-Lys-Lys-Met-Gln-Met-Lau-Lys-Lau-Asp-Lys-Glu-Asn-Ala-Lau-Asp-Arg-Ala-Glu-Ala-Lys-Lys-Ala-Ala-Glu-Asp-Arg-Ser-Cys						
C Th	40	45	50	55	60	65	70
S B-Th	-Lys-Gln-Lys-Asp-Glu-Lau-Val-Ser-Lau-Cln-Lys-Lys-Lys-Gly-Thr-Glu-Asp-Glu-Lau-Asp-Lys-Tyr-Ser-Glu-Ala-Lau-Lys-Asp-Ala-Gln-Glu-Lys-Lau-Glu-Glu						
C Th	75	80	85	90	95	100	105
S B-Th	-Lys-Ala-Glu-Lys-Lys-Ala-Thr-Asp-Ala-Glu-Ala-Glu-Ala-Ser-Lau-Asn-Arg-Arg-Ile-Gln-Lau-Val-Glu-Glu-Glu-Lau-Asp-Arg-Ala-Gln-Glu-Arg-Lau-Ala-Thr-Gln						
C Th	110	115	120	125	130	135	140
S B-Th	-Ala-Lau-Gln-Lys-Lau-Glu-Ala-Glu-Lys-Ala-Ala-Asp-Glu-Ser-Glu-Arg-Gly-Met-Lys-Val-Ile-Glu-Ser-Arg-Ala-Gln-Lys-Asp-Glu-Glu-Lys-Met-Glu-Ile-Gln-Lau						
C Th	145	150	155	160	165	170	175
S B-Th	-Glu-Ile-Gln-Lau-Lys-Glu-Ala-Lys-Met-Ile-Ala-Glu-Asp-Ala-Asp-Arg-Lys-Tyr-Glu-Glu-Val-Ala-Arg-Lys-Lau-Val-Ile-Ile-Glu-Ser-Asp-Lau-Glu-Arg-Ala-Glu-Met						
C Th	180	185	190	195	200	205	210
S B-Th	-Glu-Arg-Ala-Glu-Lau-Ser-Glu-Lys-Cys-Ala-Glu-Lau-Glu-Glu-Lys-Thr-Val-Thr-Asn-Asn-Lau-Lys-Ser-Lau-Glu-Ala-Gln-Ala-Glu-Lys-Tyr-Ser-Gln-Thr						
C Th	220	225	230	235	240	245	250
S B-Th	-Lys-Glu-Asp-Lys-Tyr-Glu-Glu-Ile-Lys-Val-Lau-Ser-Asp-Lys-Lys-Lys-Glu-Ala-Glu-Thr-Arg-Ala-Glu-Phe-Ala-Glu-Arg-Ser-Val-Thr-Lys-Lau-Glu-Lys-Ser-Thr						
C Th	255	260	265	270	275	280	285
S B-Th	-Ile-Asp-Asp-Lau-Glu-Asp-Glu-Lau-Tyr-Ala-Gln-Lys-Lys-Lys-Ala-Ile-Ser-Glu-Glu-Lau-Asp-His-Ala-Lau-Asn-Asp-Met-Thr-Ser-Ile-Lys						

Fig. 7. The amino acid sequences of cardiac and  $\beta$ -tropomyosins from rabbit. (The cardiac sequence is identical to that of  $\alpha$ -tropomyosin). Only the changes in the sequence between the two forms have been listed in the case of  $\beta$ -tropomyosin. Where the sequences are identical, a dash is shown.

(From Mak et al., 1979).

of regulation, whereby tropomyosin is envisaged to roll closer into the F-actin grooves upon activation of the thin filament by calcium. It is not unlikely then, that there could be two separate sets of binding sites on tropomyosin for the F-actin monomers, one in the "off" (inhibited) state, and one in the "on" (active) state.

The tropomyosin sequence was analysed by several laboratories (Stone et al., 1974; Parry, 1975, McLachlan and Stewart, 1976) in order to find significant repeats of amino acids which could represent actin binding sites. Distinct repeating bands every 28 Å had been observed by Parry and Squire (1973) on uranyl acetate stained Tm paracrystals. This distance corresponded to the axial separation between F-actin subunits. There were 14 such sub-repeats in each of the 396 Å major periods corresponding to the length of one tropomyosin molecule.

Inspection of the sequence (excluding those amino acids of series I and II) was undertaken in order to see if there were identical patterns of amino acids repeated seven-fold throughout the sequence which might correspond to actin binding regions. These were not found. Instead, quasi-equivalent arrangements of polar and non-polar groups of amino acids were seen instead. McLachlan and Stewart (1976a), using computer analysis refined earlier work in this area (Parry and Squire, 1973; Sodek et al., 1974) and showed that these groups were repeated fourteen times throughout the sequence and that each group consisted of 19.66 amino acid residues. Continuity was maintained by head to tail overlap of successive molecules, which allowed the repeating pattern to be carried uninterrupted over to the next tropomyosin molecule. These workers also found that acidic and non-polar residues contributed significantly to the pattern, whereas the basic residues were randomly distributed over the surface of

the coiled-coil. The fourteen quasi-equivalent actin binding regions were not entirely constant since some anomalies were seen in the Cys 190 region (residues 197-217) and in the head to tail overlap region, but on the whole they were quite similar. McLachlan and Stewart then divided these groups into two alternating sets of seven, called the  $\alpha$ -bands and the  $\beta$ -bands. As the tropomyosin molecule rolls over the F-actin surface the motion was postulated to weaken one set of interaction sites ( $\alpha$ -bands) and allow the other set ( $\beta$ -bands) to bind to a complementary site on the actin molecule (Parry, 1976; McLachlan and Stewart (1976a). Troponin would provide the torque necessary to generate such a movement. Another proposal for the occurrence of 14 actin binding sites on one tropomyosin molecule was given in the work of Wakabayashi et al. (1975). Their three dimensional image reconstructions showed that Tm could interact with both strands of the boot-shaped actin monomers in the absence of troponin, but in the presence of Tn-I and Tn-T the movement away from the groove would disrupt or weaken one set of interaction sites (homostrand) and would strengthen the other set (heterostrand). Because  $\alpha$ -bands showed more structural regularity, McLachlan and Stewart (1976a) suggested that they represented the homostrand interaction sites.

Further analysis of the sequence by Parry (1975) and Smillie et al. (1979) revealed other interesting features. The ability for each amino acid to form an  $\alpha$ -helix ( $\alpha$ -helix parameter) had been calculated by Chou and Fasman (1974). The  $\alpha$ -helix parameters for individual amino acids in the sequence were analysed by fast fourier transform techniques in order to find periodicities in the helical potential along the tropomyosin chain. The initial results were then averaged over stretches of 14 residues and were plotted as a function of the peptide bond position

in the sequence. A series of maxima and minima corresponding to each of seven 40-residue periods could be seen. The minima lined up with the positive non-polar zones of the  $\alpha$ -bands whereas the maxima corresponded to the same area in the  $\beta$ -bands. Of special interest in this work was the fact that the maxima and minima become less defined as the sequence progressed to the COOH-terminal end. This could be due to the fact that the troponin binding region may be located here (see below).

b) The Tropomyosin-Troponin Interaction

There is much historical evidence that tropomyosin and troponin (Tn) interact. The "native tropomyosin" (Ebashi and Ebashi, 1964) of early purification procedures was actually a complex of both troponin and tropomyosin (Ebashi and Ebashi, 1966). Troponin has been shown to enhance Tm viscosity (Ebashi and Kodama, 1965) and to form a faster sedimenting complex in the analytical centrifuge (Hartshorne and Mueller, 1967). Troponin has been visualized with the electron microscope at 400 A° intervals along the entire length of the thin filaments when these were labelled with ferritin conjugated anti-Tn antibodies (Ohtsuki et al., 1967). When troponin itself was conjugated to ferritin and was allowed to form paracrystals with tropomyosin, a wide white line formed the middle of the broad band normally seen in these aggregates (when stained with uranyl acetate), corresponding to the region one-third of the way from the COOH-terminal end of Tm (Nonomura et al., 1969; Ohtsuki, 1974). Troponin co-crystallizes with tropomyosin. When studied by X-ray crystallography (Cohen et al., 1972) Tn is found to be localized in the middle of the long arm of the kite-like mesh formed by the lattice of intersecting tropomyosin filaments. This area was later shown by Phillips et al. (1979) to be close to the Cys 190 residue.

McLachlan and Stewart (1976b) analysed the sequence of tropomyosin with computer techniques and found evidence near residues 197-217 for a possible Tn binding site. The outer surface of the tropomyosin coiled-coil in this region had a lower number of acidic and non-polar amino acids with respect to the rest of the molecule, making a suitable site for protein-protein interactions.

All of the data so far presented seems to indicate that Tn binds one-third of the way from the COOH-terminal end of the Tm molecule, near the Cys 190 region. However, there are data to suggest that this is an oversimplified view. When the individual subunits of troponin were tested in their ability to bind tropomyosin, only Tn-T bound (Greaser and Gergley, 1972; Margossian and Cohen, 1973) irrespective of the  $Ca^{2+}$  concentration (Potter and Gergley, 1974). When this subunit was co-crystallized with tropomyosin, a new form of lattice was seen (Greaser et al., 1972; Yamaguchi et al., 1974). It was suggested that this hexagonal lattice arose because Tn-T bound to the ends of the tropomyosin molecule, not in the troponin binding region near Cys 190.

Further evidence came from the work of Ohtsuki (1975) who prepared antibodies to all three Tn components and then added them separately to thin filaments. When viewed under the electron microscope, the anti-Tn-I and C antibodies formed narrow bands on the thin filaments whereas the antibody to Tn-T formed a much wider band and was displaced from the I and C sites towards the Z-line. Ohtsuki followed up this work (1979) by preparing antibodies to two chymotryptic fragments of Tn-T,  $T_1$  (residues 1-158) and  $T_2$  (residues 159-258). The antibody to the  $T_2$  fragment bound in the same region as the Tn-C and Tn-I antibodies, but the anti- $T_1$  antibody bound in a position 13 nm away from this area, closer to the head

to tail overlap region of the Tm molecule.

Further evidence that Tn-T may bind over a more extended region of tropomyosin (the terminal one-third) came indirectly from work by Côté et al. (1978) who showed that horse platelet tropomyosin bound weakly to troponin, although the horse platelet sequence in the region of amino acids 197-217 (the postulated Tn binding site) closely paralleled that of rabbit skeletal  $\alpha$ -Tm. The work of Pato et al. (1981) showed that Tn-T fragment CBI (residues 1-151) could enhance the head to tail aggregation of NH<sub>2</sub>-terminal and COOH-terminal fragments of  $\alpha$ -tropomyosin under conditions where they themselves did not interact. As well, Mak and Smillie (1981b) showed that whole troponin protected tyrosines 261 and 267 (which are located close to the COOH-terminal) of the tropomyosin molecule from being labelled with <sup>125</sup>Iodine.

#### F $\alpha$ AND $\beta$ -TROPOMYOSINS IN DEVELOPING AND ADULT MUSCLE

The contractile proteins of rabbit myofibrils are very well characterized to date. The sequences of five of the six major proteins have been determined; myosin being the exception. Many of these contractile proteins exist in multiple forms in vertebrate striated muscle, depending on whether the fibres are fast or slow.

Adult skeletal muscle is composed of two main types of fibres. Slow twitch fibres (type I) and fast twitch fibres (type II) can be distinguished from each other in a number of ways; histochemically (Padykula and Herman, 1955), immunologically (Dhoot and Perry, 1979), biochemically (Perry, 1974), and physiologically (Close, 1972). Transformation of the muscle proteins characteristic of one type of fibre into those of another can be accomplished, at least in part, by cross-innervation (Am-

phlett et al., 1975; Streter et al., 1975) or by chronic electrical stimulation of the intact nerve (Rubinstein et al., 1978; Roy et al., 1979b).

During muscle development, changes in the subunit patterns of the myofibrillar proteins have also been observed. The first switch is seen when myoblasts, which are the single celled precursors of muscles, stop dividing and fuse to form long multinucleated myotubes (Fig. 8). At this stage there seems to be a coordinated induction of a muscle specific program (Devlin and Emerson, 1978) which is distinct from the non-muscle proteins of the myoblasts themselves (Fine and Blitz, 1975). There is much controversy concerning the embryonic forms of these contractile proteins; do they resemble the fast types, the slow types or are they mixtures of both? This area is currently under investigation with cultured cell lines. Although easy to manipulate, these cells cannot give an accurate picture of the more complex developmental changes of the later embryonic stages, where innervation plays a vital role (Kelly and Rubinstein, 1980). Nevertheless, modern techniques such as two dimensional electrophoretic analysis of radioactively labelled synchronous cultures, fluorescent antibody labelling and analysis of single muscle fibres (Young and Davey, 1981) are making progress in a very complex field.

Tropomyosin is a good example of a polymorphic muscle protein. The ratio of  $\alpha$  to  $\beta$ -Tm is characteristic of the muscle type. Generally the slow red fibres have a higher proportion of the  $\beta$ -form than do fast white fibres. The larger, slower beating hearts of large mammals also contain some  $\beta$ -Tm while the faster beating hearts of the smaller mammals contain only  $\alpha$ -Tm (Cummins and Perry, 1974). Isoelectric focussing gels show minor subspecies of  $\alpha$  and  $\beta$ -tropomyosins ( $\alpha'$  and  $\beta'$ ) which may arise due to slight differences in their sequences (Hodges and Smillie, 1972a,b).



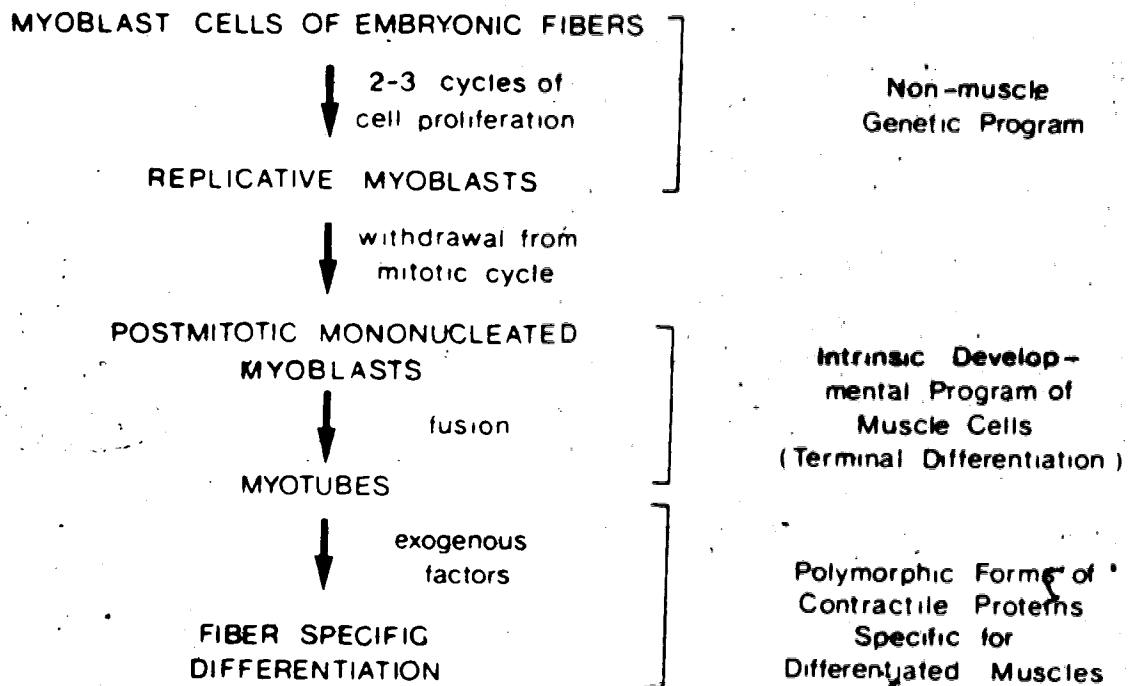


Fig. 8. A scheme for gene expression during the different phases of muscle differentiation.  
 (From Roy et al., 1978).

Tropomyosin is synthesized at the onset of myoblast fusion, and it is apparent that  $\beta$ -Tm is the major species found in the embryonic stages (Amphlett et al., 1976). In Figs. 9a and b it can be seen that  $\beta$ -Tm accounts for over 75% of the total content of tropomyosin in embryonic tissues and that the ratio changes after birth to one where  $\alpha$ -Tm is more predominant. Adult rabbit fast muscles have about 3 to 4 times more  $\alpha$  than  $\beta$ -Tm. In slow muscles the ratio is more equal (1.1 to 1). The high  $\beta$ -content in embryonic muscles argues for the existence of Tm which is composed of two  $\beta$  subunits, although to date only  $\alpha\alpha$  and  $\alpha\beta$  dimers have been observed in adult skeletal muscles (Eisenberg and Kielly, 1974; Lehrer, 1975; Yamaguchi et al., 1974).

Phosphorylation of both forms of tropomyosin has been demonstrated (Montarras et al., 1981) and this modification has been shown to be a post-translational event. The same workers have given evidence that phosphorylation is higher in the embryonic stages (in both cases). The significance of phosphorylated tropomyosin is as yet unclear.

#### G. AIMS OF THIS PROJECT

$\alpha$  and  $\beta$ -tropomyosins have been the subject of investigations in the early 1970's by Cummins and Perry (1973, 1974). Since that time both forms have been sequenced (Stone et al., 1974; Mak et al., 1979) and much progress has been made in all areas of the muscle field, especially with regards to the thin filament proteins and their interactions. The purpose of the present investigation was to study the interactions of  $\alpha$  and  $\beta$ -tropomyosins with the other members of the thin filament (actin and troponin).

Once these parameters were established we hoped to compare the biological activities of both tropomyosins in a reconstituted muscle system

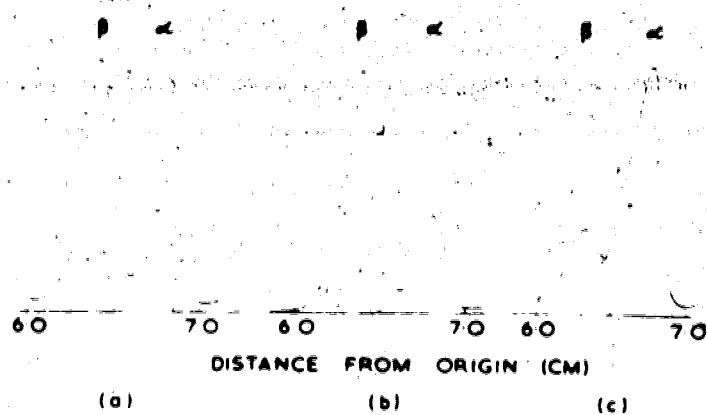
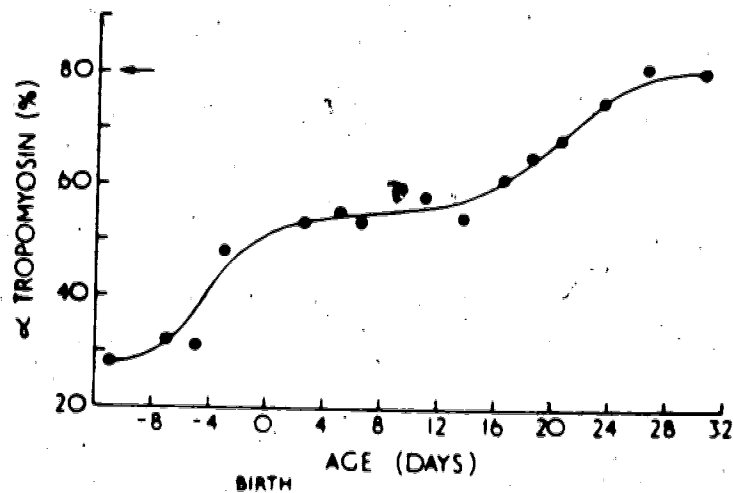


Fig. 9a. The relative proportions of  $\alpha$  and  $\beta$ -tropomyosins in the longissimus dorsi muscle of developing rabbit. Before birth  $\beta$ -Tm is the predominant form whereas shortly after birth the  $\alpha$ -form becomes more expressed.

Fig. 9b. Densitometric scans of various tropomyosin samples run on SDS-PAGE:

- a) from 20 day whole foetus
  - b) from 24 day foetus (longissimus dorsi)
  - c) from 30 day old rabbit (longissimus dorsi)
- (From Amphlett et al., 1976).

using the proteolytic myosin fragment S-1. Variations in the actin-activated ATPase activity of S-1 with one or the other form of tropomyosin present should give us a quantitative estimate of how they behave as regulatory proteins and would perhaps give us some insight as to why  $\beta$ -Tm is the predominant form in embryonic muscle.

## CHAPTER II

## MATERIALS AND METHODS

## A. PROTEIN PURIFICATIONS

1. Actin

Actin was prepared in two stages. The first consisted of making an acetone powder from rabbit skeletal muscle (Feuer et al., 1948). One kg of frozen muscle (rabbit muscle tissue, Type I, mature, New Zealand White, Pel-Freeze) was thawed slightly and passed through a meat grinder. This mince was then subjected to a number of washes (all done at 4°C or less) followed by centrifugation in a DPR-6000 centrifuge at 4760 Xg for 10 min, or in the case of organic extractions, by squeezing the residue through four layers of cheese cloth. The following steps list the extractions and their order of execution:

- 1) 12 vol distilled water, 30 min with stirring
- 2) 3 vol 50 mM Na carbonate, pH 8.0
- 3) 3 vol 0.2 mM CaCl<sub>2</sub>
- 4) 2X3 vol 95% ethanol (0°C)
- 5) 3X3 vol acetone (-20°C)

The final residue was air dried overnight on filter paper in a fumehood and was subsequently stored at -20°C. Yields were approximately 135 g powder per 1 kg muscle.

The second stage in making actin followed the method of Spudich and Watt (1971) except that the actin was separated from the muscle residue by filtration through Whatman #1 paper, followed by passage through a 0.8 µm Millipore filter.

Purified G-actin was stored at 4°C and was used within 2 weeks. Typ-

ical yields were 150 mg actin per 10 g acetone powder.

## 2. Troponin

Troponin (Tn) was prepared essentially as described by Ebashi et al. (1970). The procedure, with minor modifications is summarized below. All centrifugations were for 20 min at 4760 Xg in a model DPR-6000 centrifuge.

One kg of rabbit skeletal muscle was ground in a meat grinder. This mince was first extracted with 2 vol of Guba Straub buffer (see the myosin procedure following) containing 1 mM ATP for 15 min with stirring. After centrifugation, the residue was washed once with 20 mM KCl, 2 mM  $\text{KHCO}_3$  and then twice with distilled water (centrifugations after each wash). Troponin was then extracted for 2 h with 0.6 M LiCl, 50 mM  $\text{CH}_3\text{COONa}$  pH 4.5, 2 mM  $\beta$ -MeOH and 0.2 mM PMSF. The PMSF was added to inhibit proteolytic enzymes. After centrifugation, the supernatant was adjusted to a pH of 7.2 with 1 N NaOH and was stirred for 30 min. Then the pH was dropped to 4.5 a second time with 1 N HCl in order to isoelectrically precipitate any remaining tropomyosin. If the solution had any cloudiness in it, it was centrifuged once more. The clarified supernatant containing troponin was then subjected to three ammonium sulphate fractionations which were performed at a pH of 7.2; 0-40%, 40-50% and 50-60%. Pellets from the last two cuts were redissolved in distilled water and were dialyzed against 4X20 l changes of 2 mM  $\beta$ -MeOH. Dialysis time was kept to a minimum (24 h) in order to reduce proteolysis. Troponin was stored as a lyophilized powder at  $-20^\circ\text{C}$ .

## 3. Tropomyosin

Tropomyosin (Tm) was prepared in two stages. The first stage was the preparation of rabbit skeletal muscle powder according to the method of

Bailey (1948), the only modification being the substitution of acetone instead of ether in the final step. The muscle powder was air dried and stored at  $-20^{\circ}\text{C}$ . For preparing cardiac tropomyosin powder, rabbit hearts (Type 1, New Zealand White, mature, Pel-Freeze) were used as starting material.

Extraction of tropomyosin from the muscle powders followed the method of Bailey (1948) with modifications as described by Pato (1978). During the final ammonium sulphate fractionations, cardiac tropomyosin was obtained in the 55-65% cut whereas skeletal tropomyosin was cut slightly lower at 53-65% in order to obtain higher yields.

Cardiac Tm was further purified on a DEAE cellulose column to remove any contaminating nucleotides (Hodges et al., 1972a).

#### 4. Separation of Tropomyosin Subunits

The procedure for separating rabbit skeletal tropomyosin into its  $\alpha$  and  $\beta$ -subunits was developed by Cummins and Perry (1973). This procedure called for prior carboxymethylation of the tropomyosin, a step we wished to avoid. Instead we developed a method for quantitatively reducing the sulphhydryls so that the two subunits would behave as monomers in 8 M urea. The following steps were all done at room temperature.

Lyophilized tropomyosin (400 mg) was dissolved in 30 ml of column buffer (50 mM Na formate pH 4.2, 8 M urea, 1 mM EDTA) with stirring. The pH was brought up to 8.0 with 1 N NaOH and was allowed to stabilize. Then 0.78 ml of concentrated  $\beta$ -MeOH was added (360 mM final concentration) to the solution and stirring was continued for 3 h longer under nitrogen. After this time the pH was brought down to 4.2 and the protein was dialysed overnight against column buffer containing 5 mM DTT.

Reduced subunits were loaded onto a CM-32 column ( $d=2.5$  cm,  $h=24$

cm) which had been previously pre-equilibrated in the same DTT containing buffer. A 2.8 l gradient from 0 to 0.2 M NaCl was applied and gave the elution profile seen in Fig.10. The  $\beta$ -tropomyosin peak which eluted first occasionally has a leading shoulder, the composition of which has been shown to be  $\beta$ -subunit (SDS-PAGE in 6 M urea, see Fig. 10). It is possibly formed due to oxidation of the sulphydryls on the column thus forming  $\beta,\beta$  dimers which have a different mobility than their monomer counterparts. At any rate, these fractions were discarded. The other fractions containing  $\alpha$  and  $\beta$ -subunits were pooled separately and then dialyzed extensively against distilled  $H_2O$  containing 2 ~~ml~~  $\beta$ -MeOH. The fractions were subsequently lyophilized and stored at  $-20^\circ C$ .

#### 5. Myosin and S-1

To make myosin a number of precautions were followed. Because myosin is very sensitive to trace metals, all buffers were chelexed and millipored, all dialysis tubing was boiled in 5 mM EDTA/50 mM Na bicarbonate before use, all implements were plastic or glass and were pre-cooled before use and all steps were done at  $4^\circ C$  using only  $d^3 H_2O$  (doubly deionized distilled water).

During the course of the preparations it was found that fresh rabbit muscle gave myosin with the highest activities. Thus adult New Zealand white rabbits were sacrificed and the back and leg muscles dissected to give typically about 600 g of muscle.

Three hundred g of muscle were blended in a Waring blender at top speed for 15 s with 500 ml of Guba Straub (G-S) solution (0.3 M KCl, 0.15 M  $KPO_4$ , pH 6.5, 12 mM  $MgCl_2$ , 5 mM EGTA) and 0.925 g ATP. This step was done in 2 batches. After blending, the volume was brought up to 1650 ml with more G-S solution. The final ATP concentration was then 1 mM. The



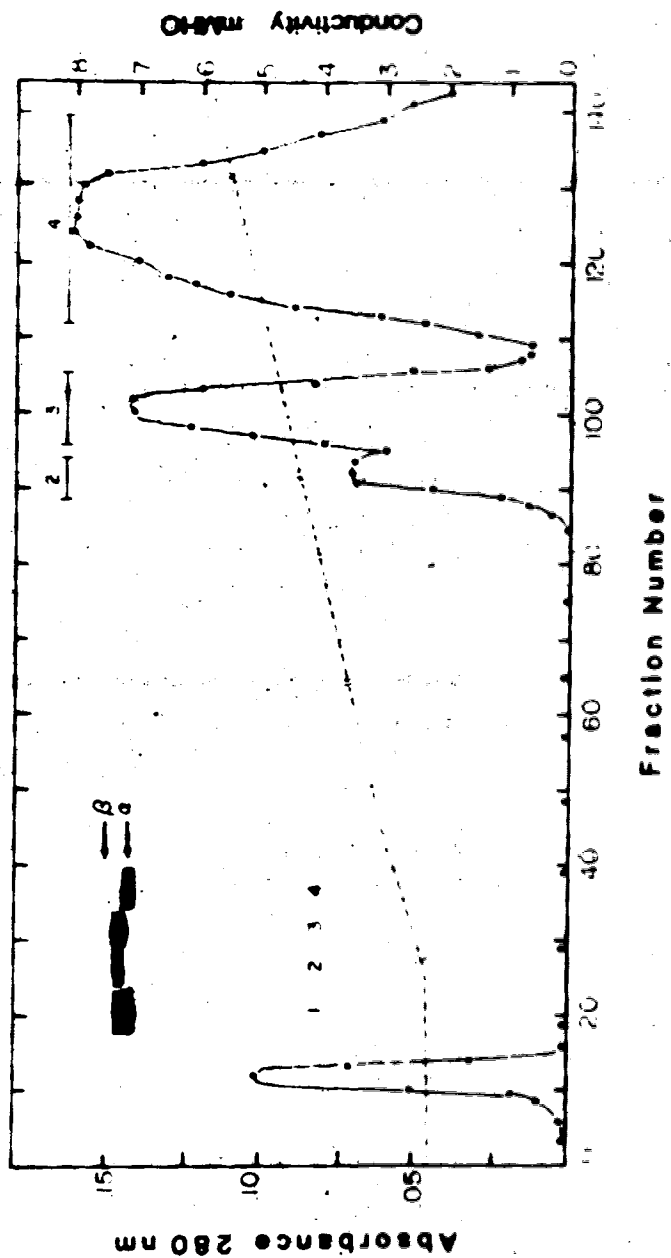


Fig. 10. CM-32 cellulose separation of  $\alpha$  and  $\beta$ -tropomyosin subunits. Gel inset fraction 1 contains rabbit skeletal tropomyosin before the column, fraction 2 contains  $\beta$ -Tm from the leading peak, fraction 3 contains  $\beta$ -Tm and fraction 4 contains  $\alpha$ -Tm.

mince was stirred for another 15 min (no longer) and then spun down for 20 min at 4760 Xg on a DPR-6000 centrifuge. The supernatant was filtered through glass wool and then added to 12 volumes of  $d^3$  H<sub>2</sub>O with stirring. This solution was then allowed to sit for 90 min so that myosin aggregates could precipitate. The supernatant was syphoned off and the myosin layer was centrifuged as above to concentrate the protein.

The opaque white myosin pellet was dissolved in 100 ml 5 X G-S solution pH 6.5 and the volume adjusted to 500 ml with H<sub>2</sub>O. ATP, pre-dissolved in 2 ml buffer, was added to give a final 1 mM concentration. This mixture was stirred gently for 15 min to dissociate any actomyosin before being spun overnight at 16,500 rpm in a J21 rotor on a model L-centrifuge. The supernatant was then filtered through glass wool to remove lipid particles and was put through 2 cycles of precipitation in 12 vol  $d^3$  H<sub>2</sub>O. Each cycle was followed by centrifugation at 4769 Xg for 20 min and resuspension in 0.5 M KCl, 1 mM EDTA, 15 mM KPO<sub>4</sub> pH 6.5. After one more precipitation as above, the myosin pellet was dissolved in a minimum amount (20-30 ml) of 2.5 M KCl, 5 mM EDTA, 75 mM KPO<sub>4</sub> pH 6.5 using a rubber policeman. The mixture was thick enough to trap air bubbles. ATP pre-dissolved in 1 ml of the buffer was added to give 1 mM final concentration. After 15 min with occasional mixing, the myosin was spun at 27,000 rpm for 2 h in an L-30 rotor on a model L-centrifuge. This step removes any actomyosin or denatured myosin. The supernatant was then dialysed against 2-4 l changes of 0.12 M NaCl, 1 mM EDTA, 0.02 M NaPO<sub>4</sub> pH 7.0. This is the "digestion buffer" (Weeds and Taylor, 1975) in which myosin is only sparingly soluble. After dialysis the thick, opaque solution was allowed to come to room temperature. The optical density of the protein was taken by making 1:10 and 1:20 dilutions in 0.6 M KCl. At this ionic strength the myosin is soluble and the A<sub>280</sub> may be taken.

The concentration of myosin was adjusted with dialysis buffer to give 15 mg/ml. Chymotrypsin was pre-dissolved in dialysis buffer and was added to the myosin to give a 0.05 mg/ml final concentration. Stirring was brisk during the digestion period (15 min). The reaction was quenched with 1 mM DFP (caution) and the digest was centrifuged for 3 hours at 27,000 rpm in a model L-centrifuge (L-30 rotor) to bring down undigested material and the insoluble rod portion of the myosin molecule.

The supernatant containing the globular myosin heads (S-1 fragments) was then dialysed against 3X4 l changes of column buffer (50 mM Tris pH 7.9, 1 mM EDTA) and loaded onto a DEAE cellulose column (d=2.6 cm, h=41 cm) which had been pre-equilibrated in the same buffer. S-1 was eluted from the column with a 1500 ml gradient from 0 to 0.2 M NaCl. The elution profile (Fig. 11) gave two peaks which corresponded to S-1 with either the A1 or the A2 light chain attached. Both fractions were pooled together and concentrated to 10 mg/ml in an Amicon pressure cell using a PM-30 membrane.

Activity of the S-1 was checked using the  $K^+$ -EDTA ATPase assay (see details in section D of materials and methods). Activities were generally 6.5  $\mu\text{moles PO}_4/\text{min/mg S-1}$  ( $13 \text{ s}^{-1}$ ) in preparations from fresh rabbit muscle. Published values vary from 7.3 to  $13 \text{ s}^{-1}$  (Margossian and Lowey, 1978; Shrivvers and Sykes, 1981). Typical yields were 600-650 mg S-1 per 300 g skeletal muscle. The S-1 stayed active for a period of 4 weeks when stored at 4°C without DTT present.

#### 6. Non-Polymerizable Tropomyosin (NPTm)

Non-polymerizable tropomyosin was a generous gift from Dr. Alan Mak and was prepared according to the published procedure in Mak and Smillie (1981a).

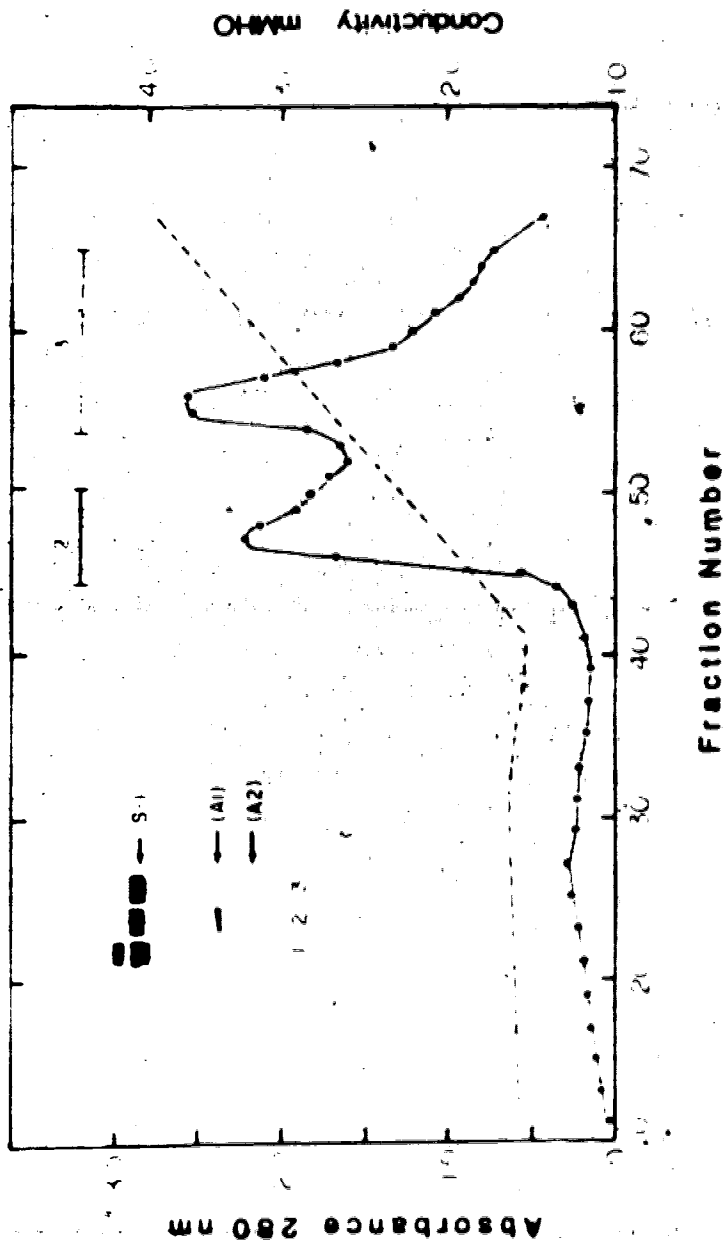


Fig. 11. DEAE-52 cellulose purification of rabbit skeletal S-1. Gel inset fraction 1 contains unfractionated myosin S-1 digest, fraction 2 contains S-1 containing only the A1 light chain and fraction 3 contains S-1 and the A2 light chain.

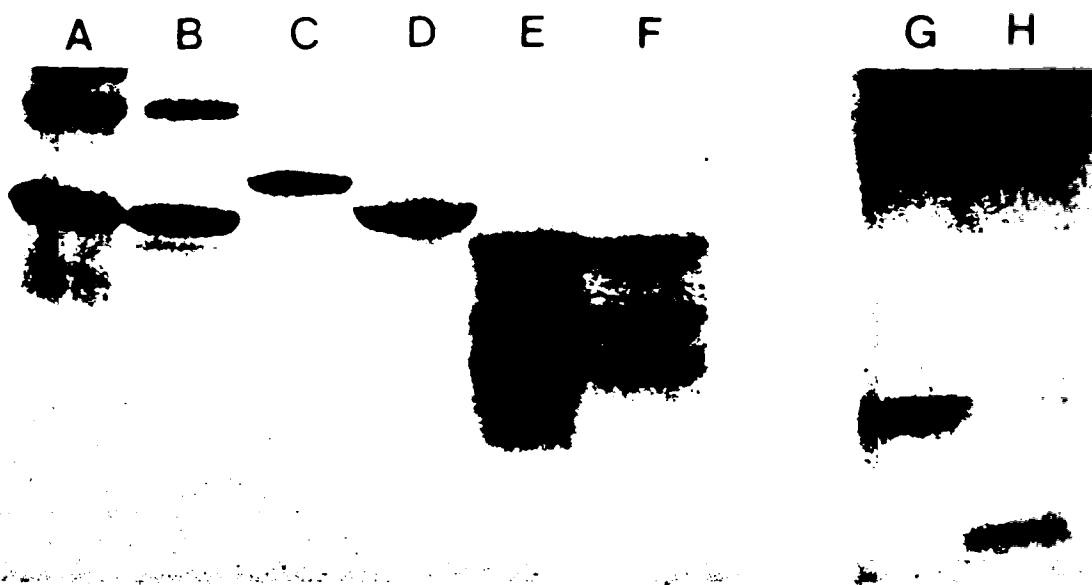


Fig. 12. The proteins used for the experiments in the following chapters;

- A) Cardiac tropomyosin
- B)  $\alpha$ -tropomyosin
- C)  $\beta$ -tropomyosin
- D) Actin
- E) Troponin used in chapter 4
- F) Troponin used in chapter 3
- G) S-1·A1
- H) S-1·A2

Note: the S-1·A1 and S-1·A2 fractions were pooled before use.

## 7. Cyanogen Bromide Fragment C81 of Troponin T

Troponin T was isolated from the troponin complex as is described in Greaser and Gergely (1971). The cyanogen bromide fragment C81, which encompasses residues 1 to 151 of Tn-T was prepared according to the method of Pearlstone et al. (1977).

## B. PROTEIN INTERACTION STUDIES

### 1. Viscosity measurements

All viscosity measurements were done with a Cannon-Manning semi-micro type A50 viscometer. Solvent flow-through-time was approximately 100s. Temperature was kept constant at  $20 \pm 0.1^\circ\text{C}$  with a circulating water bath. Between runs the viscometer was cleaned with soapy water, distilled water, ethanol and air-dried with acetone. The flow through time for each solution was measured three times and the average used to calculate  $\eta_{rel}$ .

For head to tail polymerization studies, lyophilized tropomyosin was dissolved in the appropriate buffer (10 mM cacodylate pH 7.0 and varying amounts of KCl, from 0 to 0.2 M) containing 100 mM  $\beta$ -MeOH. This was necessary to pre-reduce the sulphhydryls before overnight dialysis against the same buffer, but with only 2 mM reducing agent present. Proteins were clarified by centrifugation in a desk top centrifuge before being adjusted to 2 mg/ml by absorbance. Samples of 0.5 ml were introduced into the viscometer, the temperature was allowed to equilibrate for 5 min and the measurements were then taken.

For the troponin-tropomyosin interaction studies the same procedure was followed but the tropomyosin was adjusted to 1 mg/ml and the troponin to 2 mg/ml. For each run a constant amount of Tm was used (0.25 mg) whereas the troponin varied between 0 to 0.5 mg in the assays. The volume was

adjusted to give 0.5 ml with dialysis buffer. Stock proteins were kept on ice to reduce the danger of proteolysis (especially of troponin).

## 2. Gel Filtration

Both forms of tropomyosin and the Tn-T fragment CBI were run alone or in combination on a Bio-Gel A 0.5m column ( $d=1.2$  cm,  $h=90$  cm) which was equilibrated with 10 mM imidazole pH 7.0, 1 mM DTT, 0.01% Na azide and varying amounts of KCl (0.1 to 0.25 M). The tropomyosin was dialysed overnight at 4°C in the buffer of choice. Each run required 250  $\mu$ l of a 2 mg/ml solution (0.5 mg; 7.6  $\mu$ mol). The CBI was made up as a stock 2.74 mg/ml solution in buffer containing 0.1 M KCl. 100  $\mu$ l aliquots were frozen until just prior to use (0.274 mg; 15.2  $\mu$ mol). Then they were thawed, adjusted to the proper ionic strength by adding tiny amounts (depending on the conditions of the run) of 1 M KCl and mixed with the tropomyosin. The proteins were incubated for 15 min before being applied to the column.

Elution proceeded at 7.3 ml/h and 1.8 ml fractions were collected. These were monitored using the dye binding assay (section C, this chapter) at 595 nm. Selected fractions were run on SDS-PAGE in 6 M urea to visualize the extent of protein associations.

## 3. Affinity Chromatography

Two g of CNBr activated Sepharose 4-B (Pharmacia) was allowed to swell overnight in 1 mM KCl at 4°C. The next day it was filtered and coupled to whole troponin (21 mg/11 ml) which had been dissolved in 0.1 M  $\text{NaHCO}_3$ , 0.5 M NaCl pH 8.0. There was continuous end over end mixing during the 2 h reaction time. This step and the ones following were done at room temperature.

After coupling, the resin was again filtered and washed with 200 ml of coupling buffer on a sintered glass funnel. Troponin bound to the

beads was estimated by subtracting the  $A_{280}$  of the effluent from the initial absorbance. The coupling efficiency was approximately 97%.

The resin was now treated with 15 ml of 1 M ethanolamine pH 8.0 for 1.5 h with continuous and over end mixing in order to deactivate any remaining CNBr groups on the gel matrix. The Tn-Sepharose was then filtered on a sintered glass funnel and subjected to alternating washed (3 times each) of 1 M NaCl, 0.1 M Na acetate pH 4.0 and 1M NaCl, 0.1 M Na borate pH 8.0 in order to remove any non-covalently bound proteins. A small column (d=0.9cm, h=9.2 cm, v=5.8 ml) was packed with this resin and washed overnight at 4 ml/h at 4°C with column buffer (10 mM imidazole pH 7.0, 0.01% Na azide, 1 mM EGTA and 1 mM DTT).

#### 4. Actin Co-sedimentation

Actin co-sedimentation studies were based on the method of Eaton et al. (1975). Actin and  $^{125}\text{I}$ -labelled  $\alpha$  or  $\beta$ -tropomyosin (of known specific activity) were dialysed overnight against 3 mM Tris pH 7.8, 30 mM KCl, 2 mM ATP, 0.1 mM EGTA and 2 mM DTT. After dialysis, the tropomyosin was pre-spun for 1 h at 100,000 Xg to bring down any particulate matter and the actin was clarified at 3,700 Xg in a desk top centrifuge. The actin concentration was carefully determined by absorbance spectroscopy using the following formula (Johnson and Taylor, 1976):

$$\frac{A_{290} - 1.34 (A_{320})}{0.69} = \text{mg/ml}$$

In one set of experiments, KCl was varied between 30 and 400 mM and in the other set, the magnesium concentration was altered (0 to 12 mM). Otherwise the co-sedimentation conditions were unchanged. In each 1 ml sample the actin and tropomyosin were present in a 7/2 ratio (10  $\mu\text{M}$



to 2.8  $\mu\text{M}$ ), vortexed once briefly and incubated at room temperature for 15 min. Two 100  $\mu\text{l}$  samples were taken before and after a 90 min centrifugation run at 100,000 Xg and 20°C. These samples were counted in an LKB 1270 Rackgamma II  $\gamma$ -ray counter and the difference between the two groups was taken to represent the amount of tropomyosin that had pelleted (associated) with the actin under the experimental conditions. Corrections were made to account for the decay of  $^{125}\text{I}$  iodine, which has a half-life of 60.2 days.

### C. PROTEIN CONCENTRATION DETERMINATIONS

#### 1. Absorbance Spectroscopy

Protein concentrations were routinely determined using a Gilford 240 spectrophotometer and the extinction coefficients and molecular weights given in the table below:

Protein	$E_{280}^{1\% 1\text{cm}^{-1}}$	Reference	M.Wt.	Reference
Actin	11.0	Houk and Ue (1974)	42,000	Elzinga <u>et al.</u> (1973)
Myosin	5.88	Verpoorte and Kay (1966)	470,000	Lowey and Cohen (1961)
S-1	7.9	Yagi <u>et al.</u> (1967)	115,000	Weeds and Taylor (1975)
Tropomyosin $\alpha + \beta$ subunits	3.3	Woods (1969)	65,400	Stone <u>et al.</u> (1974)
Troponin	4.7	Lowell and Winzor (1977)	69,540	Sum of parts Côté (1981)

#### 2. The Dye Binding Assay

The dye binding assay used to monitor some of the column profiles

was based on the method of Bradford (1976). The coomassie brilliant blue G-250 protein reagent (0.01% w/v dye, 4.7 v/v ethanol and 8.5% v/v phosphoric acid) can be obtained as a concentrate from Biorad. The dye concentrate was diluted 1:4 with distilled water. Equal parts of this solution and the column fractions of interest were mixed together and the  $A_{595}$  nm was read after 10 min. Colour development occurred almost instantaneously, and was stable for over 1 h.

### 3. Amino Acid Analysis

Amino acid analysis was occasionally used to determine protein concentrations. These were done on a Durrum model D-500 or a Beckman 120 C analyser which automatically integrated the peak areas of the amino acids.

Samples of proteins were hydrolysed in evacuated, sealed tubes with constant boiling HCl and 0.1% phenol. Tubes were incubated at 110°C for 24 h, the seal was broken and the liquid was dried down in a desiccator over NaOH pellets. The proteins were redissolved in a known amount of running buffer and aliquots were loaded on the analyser. Values were obtained for Ala and Leu. With a knowledge of these amino acid contents in the proteins, the protein molecular weight and the dilution factors used, it was possible to obtain accurate estimates of the protein concentrations.

## D. MYOSIN (S-1) ATPASE METHODS

### 1. The $Mg^{2+}$ -dependent, Actin-activated Myosin(S-1) ATPase

The actin-activated ATPase of myosin and its fragments HMM or S-1 (hereafter referred to as the myosin or S-1 ATPase) was measured essentially as described by Côté (1980). Proteins for the assay were prepared the day before as follows: G-actin, S-1 and tropomyosin were dialysed

overnight at 4°C against 30 mM KCl, 2 mM Tris pH 7.8, 5 mM MgCl<sub>2</sub>, 0.1 mM EGTA and 2 mM DTT. Troponin was dialysed in the same buffer without EGTA. The proteins were clarified by centrifugation the next day and the concentrations determined by absorbance. Serial dilutions were made in order to ensure accuracy.

ATP (0.2 M) was made by dissolving 0.22 g disodium ATP in small aliquots of 0.5 M KOH until the pH was 7.8. The volume was then made up to 2 ml with assay buffer.

Dilute KOH (10 mM) titrant was made from a stock 0.5 M solution which had been standardized against potassium hydrogen phthalate (Fischer Primary Standard).

Because DTT caused significant electrode drift, it was omitted from the actual assays. These were conducted in the following manner; proteins were pipetted into glass vials and assay buffer was added to give 2 ml final volume. The vials were put into a 25°C water bath to equilibrate before being transferred to a glass water jacket which maintained the temperature at 25°C during the course of the experiment.

The pH was measured with a Radiometer GK2321c electrode. Any drop in the pH due to ATP hydrolysis resulted in a signal from the electrode to the Radiometer TTT-2 Titrator which in turn released the KOH titrant into the assay medium via a SBULa syringe pipette. This compensation was recorded with a Titrigraph and the results were used to calculate the rate of ATP hydrolysis.

Under these assay conditions the S-1 had a small but measurable activity of its own. This value was subtracted from the actin-activated values when the contribution was greater than 5%.

## 2. The $K^+$ -EDTA ATPase

Samples of myosin or S-1 were dialysed for 2 h at 4°C against 2 mM Tris pH 7.8, 0.6 M KCl, 5 mM EDTA and 2 mM DTT. Protein aliquots were assayed in 2 ml 0.6 M KCl, 5 mM EDTA at 25°C following the procedures outlined in the previous section.

## E. OTHER METHODS

### 1. Poly Acrylamide Gel Electrophoresis

All gels in this thesis were run on a slab gel apparatus using the method of Sender (1971), which gives maximum resolution of the tropomyosin subunits. Samples were dissolved in 6 M urea, 50 mM  $KPO_4$  pH 7.0, 1% SDS  $\beta$ -MeOH (1%), bromophenol blue (1% of a 0.5 % solution in 0.1 M  $KPO_4$  pH 7.0) and 5% glycerol. The samples were incubated for 1 h at 60°C.

After the electrophoresis was complete, the gels were soaked for 1 h in 10% MeOH and 10% HOAc (destaining solution) to remove excess SDS and were then stained for 45 min in 0.25% coomassie brilliant blue R-250 in 50% MeOH and 10% HOAc. Destaining was done overnight or longer.

Occasionally gels were cut into strips and scanned at 595 nm on a Gilford spectrophotometer with a gel scanning attachment and recorder.

### 2. Radioactive Iodination of Tropomyosin Subunits

The method for radioactively labelling tropomyosin with  $^{125}$  iodine followed the procedure of Eaton et al. (1975). Lyophilized proteins (40 mg) were dissolved in 2 ml of 0.4 M KCl, 50 mM  $KPO_4$  pH 7.0 and dialysed to remove traces of reducing agents. The solutions were then transferred to small capped vials to which 0.1 mg lactoperoxidase (Sigma) was subsequently added (Morrison, 1974). The radioactive iodine (20  $\mu$ l of Na  $^{125}$ I; carrier free, 17 Ci/mg, 50 mCi/ml, New England Nuclear) was first mixed

with 10  $\mu$ l  $\text{KPO}_4$  pH 7.0 and 30  $\mu$ l 10  $\mu\text{M}$   $\text{Na}_2\text{SO}_3$ . Half of this mixture (30  $\mu$ l; 0.5 mCi) was transferred to each vial. The reaction was initiated with 10  $\mu$ l 0.03%  $\text{H}_2\text{O}_2$  and stirring was maintained throughout the time period. The same amount of  $\text{H}_2\text{O}_2$  was added six more times at intervals of 10 min. The fifth time, 10  $\mu$ l of 0.005 M KI was added along with the hydrogen peroxide. Ten min after the final addition, 40  $\mu$ l of 50 mM DTT was added to quench the reaction.

Proteins were dialysed against 4X1 l changes of 0.1 M KCl, 0.5 mM DTT and 50 mM  $\text{KPO}_4$  pH 7.0 at 4°C. After this time they were centrifuged in order to remove any particulate matter. Samples of the supernatant were counted (LKB 1270 Rackgamma 11  $\gamma$ -ray counter) to determine the extent of labelling and the specific activity of the proteins.

Stock solutions of proteins were frozen in smaller aliquots in glass vials which were stored in lead containers at -20°C.

CHAPTER III  
INTERACTION STUDIES WITH  $\alpha$  AND  $\beta$ -TROPOMYOSINS

Tropomyosin is a structural protein and has no enzymatic activity with which it can be quantified. Thus in order to measure Tm's biological properties one must investigate its ability to interact with itself (in a head to tail manner) and with the other proteins of the thin filament, namely troponin and actin. In these experiments we set out to distinguish the differences, if any, between  $\alpha$  and  $\beta$ -tropomyosins with respect to these criteria. The availability of both sequences allowed us to interpret the results.

Fig. 7 illustrates the primary structure of rabbit skeletal  $\beta$  and cardiac tropomyosins. Since cardiac Tm and skeletal  $\alpha$ -Tm have the identical sequence (Lewis and Smillie, 1980) this figure can be used for our purpose of comparison. Out of 284 residues, there are 39 substitutions between the two forms. Most of these (23) are situated on the surface of the molecules (sites for protein-protein interactions) and three occur in the last 9 residues of the chain (the head to tail overlap region). The bulk of the substitutions involve exchanges with chemically similar amino acids; however two replacements (Ser<sub>229</sub> to Glu and His<sub>276</sub> to Asp) result in a more net negative charge for the  $\beta$ -form, allowing the two tropomyosins to be separated on CM-cellulose (Fig. 10). The repeating pattern of polar and non-polar amino acids characteristic for coiled-coils has been preserved in the  $\beta$ -sequence. In addition, the g-e ionic interactions are mostly conserved with the exception of the Ser<sub>229</sub> to Glu exchange. This glutamic acid residue will experience electrostatic repulsion from Glu<sub>224</sub> (position g') from the other  $\alpha$ -helical chain. This may lead to local destabilization of the coiled-coil (Mak et al., 1980).

With this knowledge to start with, it became interesting to see if the biological properties of the two forms of Tm were similar.

#### A. HEAD TO TAIL POLYMERIZATION STUDIES

The head to tail polymerization property of tropomyosin molecules can be effectively studied with the technique of viscosity. At low ionic strengths polymerization is most apparent (Tsao and Bailey, 1953; Kay and Bailey, 1960), but it diminishes as the salt concentration is increased. The extent of viscosity is also dependent on pH and temperature (Iida and Ooi, 1967).

McLachlan and Stewart (1975) proposed a model for this head to tail association. They suggested that the tropomyosin overlap of 9 amino acid residues would occur by an interlocking of the flat broad faces of the two coiled-coils (one from the COOH-terminal of one molecule and one from the NH<sub>2</sub>-terminal of another). This pairing would allow for the most favourable hydrophobic and electrostatic interactions between the two molecules and would effectively double the thickness of the filaments in the overlap region. Met<sub>281</sub> was postulated to play a major "spacefilling role" in the hydrophobic core of the two interacting supercoils.

Johnson and Smillie (1977) studied the ability of  $\alpha$  and  $\beta$ -tropomyosins to polymerize while undergoing enzymatic digestion with carboxypeptidase A (a COOH-terminal exopeptidase). Their results showed that in  $\alpha$ -Tm polymerizability was lost only after Met<sub>281</sub> had been removed, in agreement with the model above. In  $\beta$ -tropomyosin Met<sub>281</sub> is replaced by an isoleucine. The similar digestion profiles for the two tropomyosins was an indication that Ile plays a similar spacefilling role as Met (both have rather bulky sidechains). In other words, the Met<sub>281</sub> to Ile replacement is not expected to affect  $\beta$ -Tm's ability to polymerize.

Two other amino acid substitutions occur in the head to tail overlap regions (see Fig. 13); His<sub>276</sub> to Asn and Ile<sub>284</sub> to Leu. His<sub>276</sub> has been postulated by McLachlan and Stewart to form a salt bridge with Asp<sub>2</sub> in the amino terminal region of the neighbouring molecule. This role may not be fulfilled by the Asn replacement in  $\beta$ -Tm as it has an uncharged side chain. Finally, the terminal Ile has been proposed to link ionically with Lys<sub>6</sub> via its charged carboxyl group. This role could be maintained by Leu in the COOH-terminal position.

Overall then, of three amino acid differences between the two subunits, only one should theoretically lead to destabilization of head to tail interactions, the His<sub>276</sub> to Asn substitution. McLachlan and Stewart's theoretical model, however, is challenged by the crystallographic evidence of Phillips *et al.*, (1979) which shows only a slight broadening of the tropomyosin molecule in the region of overlap (instead of the double thickness predicted by the previous model). These researchers proposed that the head to tail region may consist of globular domains. How amino acids would relate to one another in these domains is not easily predictable.

Fig. 14 shows that  $\alpha$ ,  $\beta$  and cardiac tropomyosins all have the same viscosity profiles. Viscosity in all three cases is highest at the lower ionic strengths. McCubbin and Kay (1969) had previously shown that Tm structure does not alter as a function of the salt concentration, so we may interpret our results on the basis of head to tail polymerization. Rabbit cardiac tropomyosin was used as a control since it has the same sequence as the skeletal  $\alpha$ -form, but had not been subjected to 8 M urea during its purification. Cardiac Tm had the same behaviour as the skeletal tropomyosins at all ionic strengths studied. The technique of viscosity



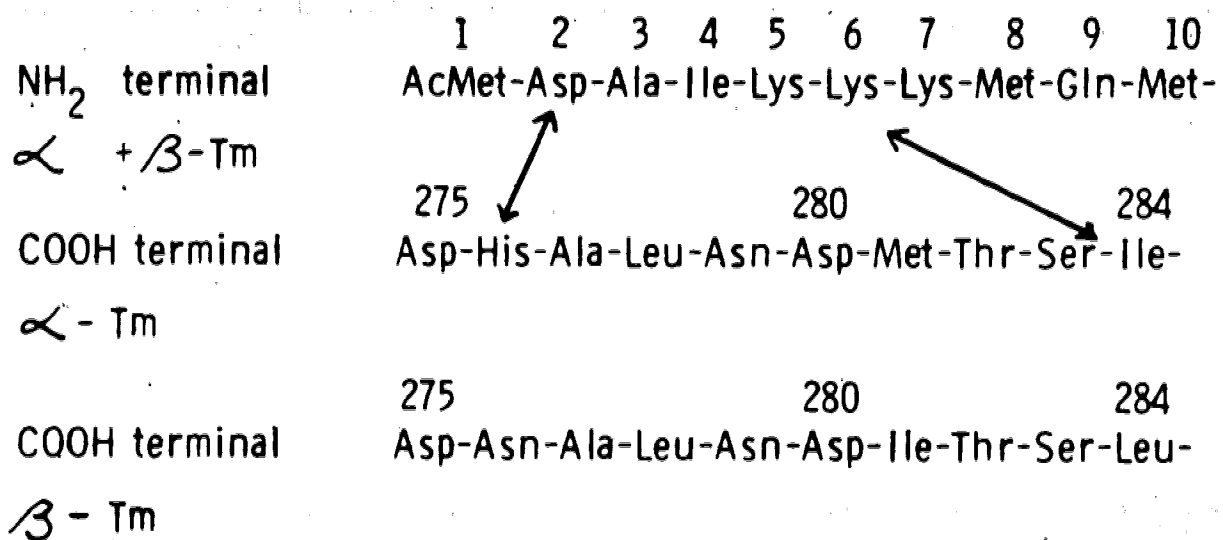


Fig. 13. NH<sub>2</sub> and COOH-terminal sequences of  $\alpha$  and  $\beta$ -tropomyosins. The double headed arrows illustrate the His<sub>276</sub> to Asp<sub>2</sub> and the Ile<sub>284</sub> to Lys<sub>6</sub> ionic interactions which are discussed in the text.

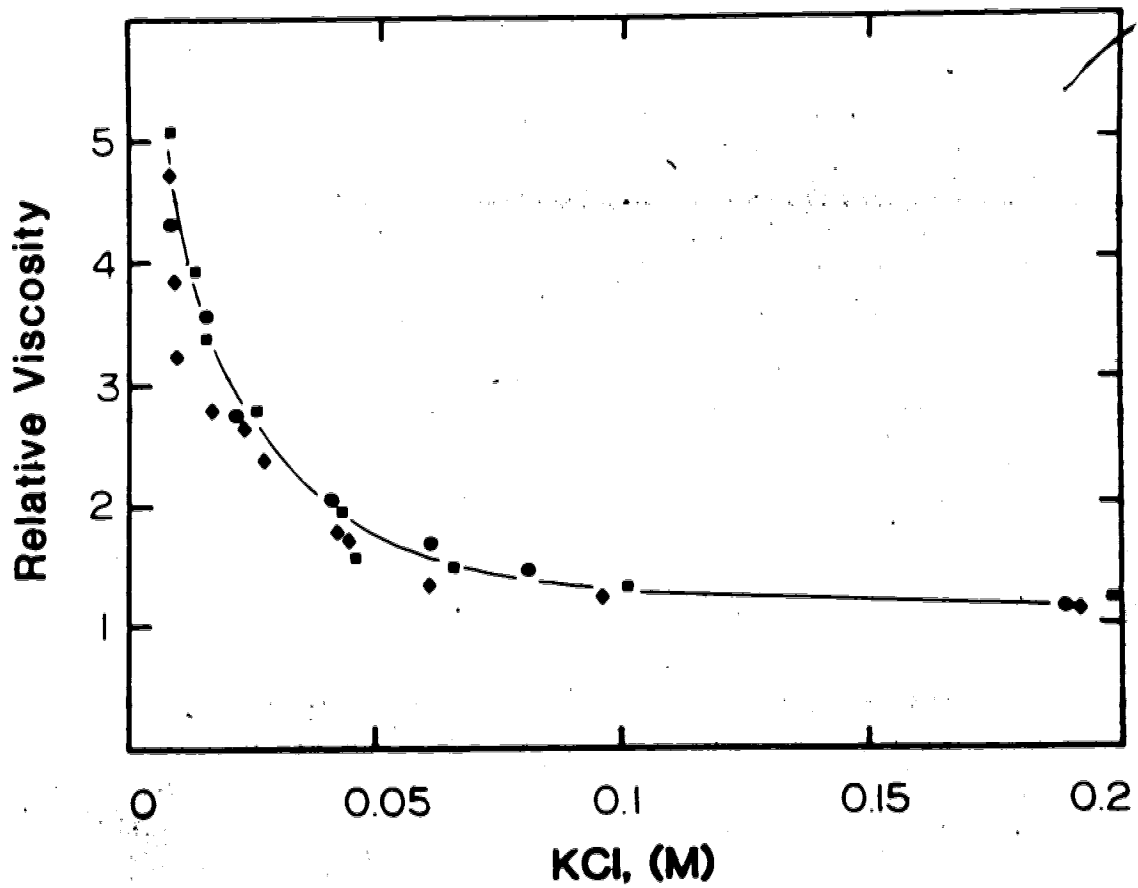


Fig. 14. A plot of the relative viscosity for skeletal  $\alpha$ -Tm ( $\bullet$ ), skeletal  $\beta$ -Tm ( $\blacklozenge$ ) and cardiac Tm ( $\blacksquare$ ) as a function of ionic strength. Tm concentration was 2 mg/ml.

indicates that there is no difference between the various tropomyosins in their abilities to polymerize in a head to tail manner.

## B. TROPOMYOSIN-TROPONIN INTERACTIONS

An indication that troponin binding may be altered between the two types of tropomyosin came from a study by Mak et al., (1980) in which the smoothed  $\alpha$ -helical parameters for both sequences were calculated in order to provide an indication of the helical periodicity of the chains. Fig. 15 illustrates the comparison.  $\alpha$ -Tm (----) shows seven periods of about 40 residues each repeated in the course of the 284 amino acids. The maxima and minima can be aligned with the  $\alpha$  and  $\beta$ -bands (respectively) below the profiles, which are the putative actin binding zones (McLachlan and Stewart, 1976). The  $\beta$ -Tm profile (.....) is similar to that of  $\alpha$ -Tm but the differences between them are accentuated in the COOH-terminal region (the area where troponin is thought to bind), undoubtedly a reflection of the greater number of amino acid substitutions in the latter half of the molecule. This analysis, coupled with the fact that Pato (1978) observed  $\beta$ -Tm to bind less tightly to a Tn-Sepharose 4B affinity column, prompted us to investigate further.

### 1. Interaction by Viscosity

Ebashi and Kodama (1965) were the first to show that whole troponin could specifically elevate the viscosity of tropomyosin above the level of a sum of their two respective viscosities. This result could be mimicked with Tn-T (Yamamoto and Maruyama, 1973; Jackson et al., 1975) and with CB1, the soluble cyanogen bromide fragment of Tn-T (Jackson et al., 1975). Troponin may increase the viscosity of a Tm solution either by transmitting a conformational change to the ends of the molecule or by interacting directly close to the overlap junction via the Tn-T subunit.

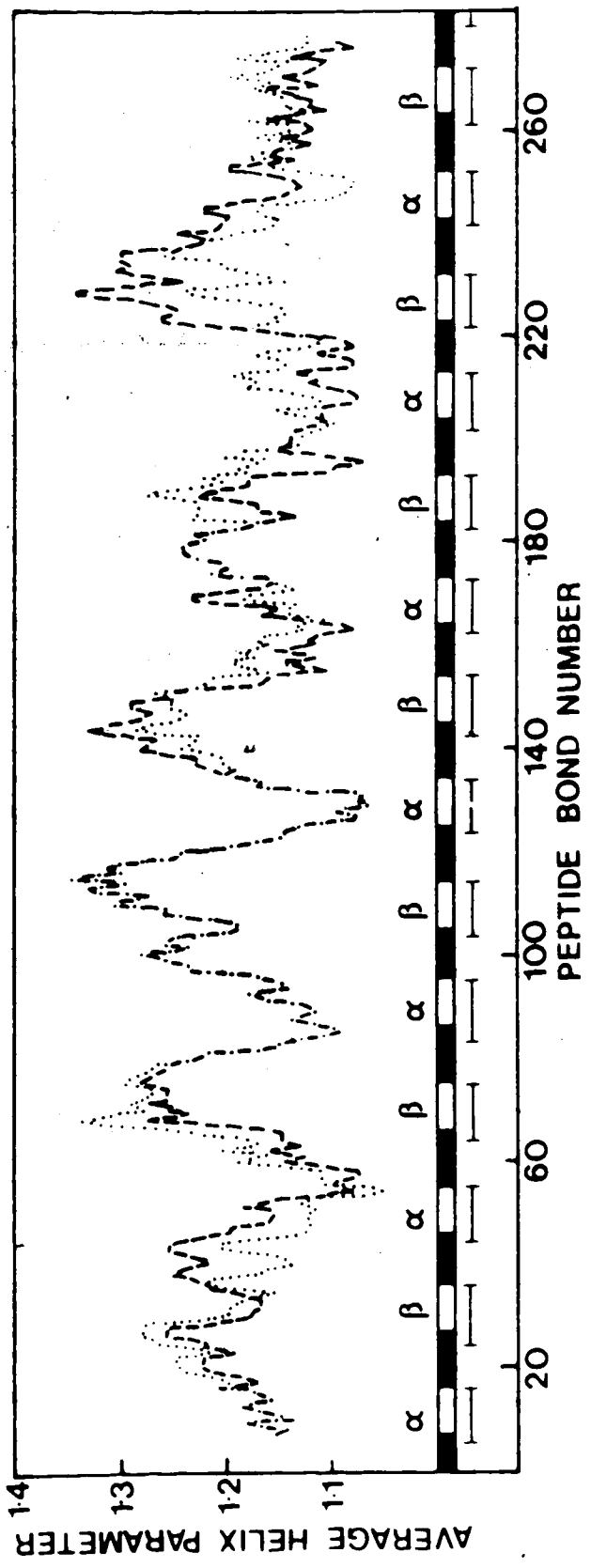


Fig. 15. A plot of the smoothed  $\alpha$ -helix parameters for  $\alpha$ (---) and  $\beta$ (.....)-Tms. The  $\alpha$ -helix parameters of individual amino acids have been averaged over 14 residues and plotted as a function of the amino acid at the center of each stretch. The maxima and minima of the profiles correlate well with the  $\alpha$  and  $\beta$ -bands (actin-binding zones) plotted below.  
(from Mak et al., 1979).

Evidence (Mak and Smillie, 1981b; Pato et al., 1981) is mounting in favour of the latter possibility.

In our studies, various amounts of whole troponin were added to  $\alpha$  or  $\beta$ -tropomyosin under diverse ionic conditions and the relative viscosities were assayed. For consistency both Tms were studied on the same day and the same troponin preparation was used to obtain the data for all the five curves. Reducing conditions were crucial for the reproducibility of the results. Thus all the proteins were pre-reduced with 100 mM  $\beta$ -MeOH and then dialysed overnight against buffers containing 2 mM DTT.

Figure 16 illustrates the results obtained. Viscosity was highest at the lower ionic strengths (0.05 M KCl) and increased as the molar ratio of Tn/Tm increased. The viscosity continued to rise well beyond a molar ratio of 1:1, a result consistent with the observations of Sugita et al., (1967). As salt was increased the relative viscosities became progressively less even though the interactions between the two molecules are reported to be not entirely abolished (Greaser et al., 1972; Hartshorne and Mueller, 1967) (see discussion). The profiles obtained show that  $\alpha$  and  $\beta$ -Tm appear to have essentially the same affinity for whole troponin in the KCl range from 0.05 to 0.25 M.

## 2. Interaction by Affinity Chromatography

Since a preliminary experiment by Pato (1978) had shown altered affinities between the two forms of Tm on a troponin Sepharose 4B column, we ventured to reproduce these results. The troponin preparation used in making the column was the same one as had been used in the previous viscosity experiments. Proteins of interest were applied to the column and were subsequently eluted with a KCl gradient. The salt concentration at which the peak of each protein eluted was taken as a measure of its

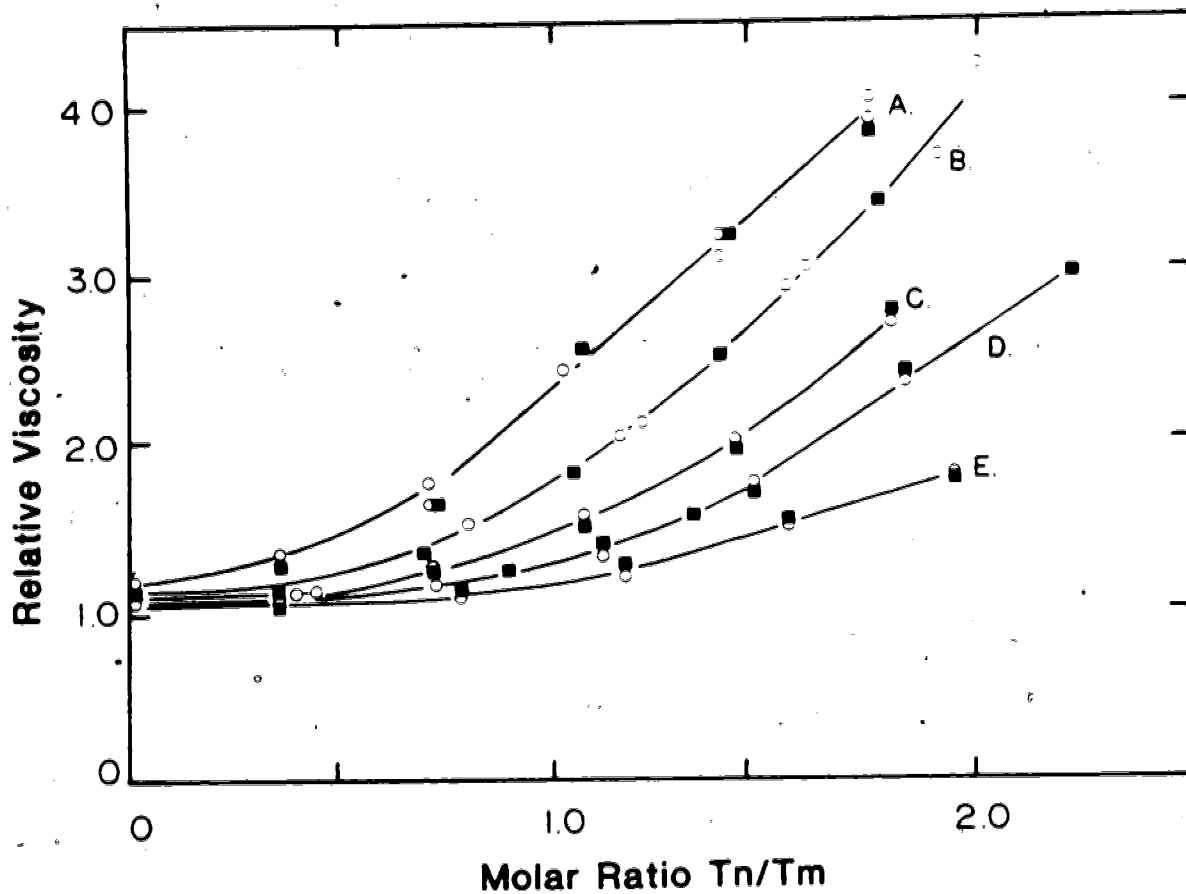


Fig. 16. Effect of the addition of skeletal troponin on the viscosity of  $\alpha$ -Tm ( $\circ$ ) and  $\beta$ -Tm ( $\blacksquare$ ) solutions. The Tm concentration was 1 mg/ml, Tn was varied. The buffer was 10 mM cacodylate pH 7.0, 2 mM  $\beta$ -MeOH and salt (KCl) to the desired concentration as indicated below:

- A) 0.5 M KCl
- B) 1.0 M KCl
- C) 1.5 M KCl
- D) 2.0 M KCl
- E) 2.5 M KCl

strength of binding for troponin. The elution profiles in Fig. 17 clearly show that bovine serum albumin, (BSA) which was used as a control, does not bind (eluted in the void volume). The two tropomyosins, however, were retained to different degrees. There was a significant and reproducible (three times each) difference in the concentration of KCl needed to elute  $\beta$ -Tm (0.12 M) and  $\alpha$ -Tm (0.2 M). This was surprising in the light of the results from the previous set of experiments, but confirmed Pato's previous observations. For this reason we chose a third technique to study this interaction.

### 3. Interaction Studies by Gel Filtration

Gel filtration studies are useful when proteins are of different shapes and sizes. Troponin and tropomyosin are dissimilar in shape; however they have approximately the same molecular weight and so the resolution between them would not be great enough for our purposes. Tn-T, a more likely candidate, introduces complications due to its insolubility at physiological ionic strengths. This leaves only CBI as a possible choice. CBI has been shown to interact with  $\alpha$ -Tm (Jackson et al., 1975; Pearlstone and Smillie, 1979; Pato et al., 1981) and it is soluble at low ionic strengths. Its approximate molecular weight of 18,000 daltons allows for good resolution from  $\alpha$ -Tm on a Bio-Gel A-0.5 m column (1.2 X 90 cm).

Our results show that CBI was eluted in fractions 30 to 34 and both the tropomyosins in fractions 22 to 27. Mixtures of CBI with  $\alpha$  or  $\beta$ -Tm gave composite profiles (Figs. 18, 19, 20 and 21), depending on the ionic strengths of the running buffers. At low KCl concentrations (0.1 M) CBI associated strongly with both forms of Tm and co-eluted in the earlier fractions (Fig. 18). Even here a small amount of CBI eluted at the nor-

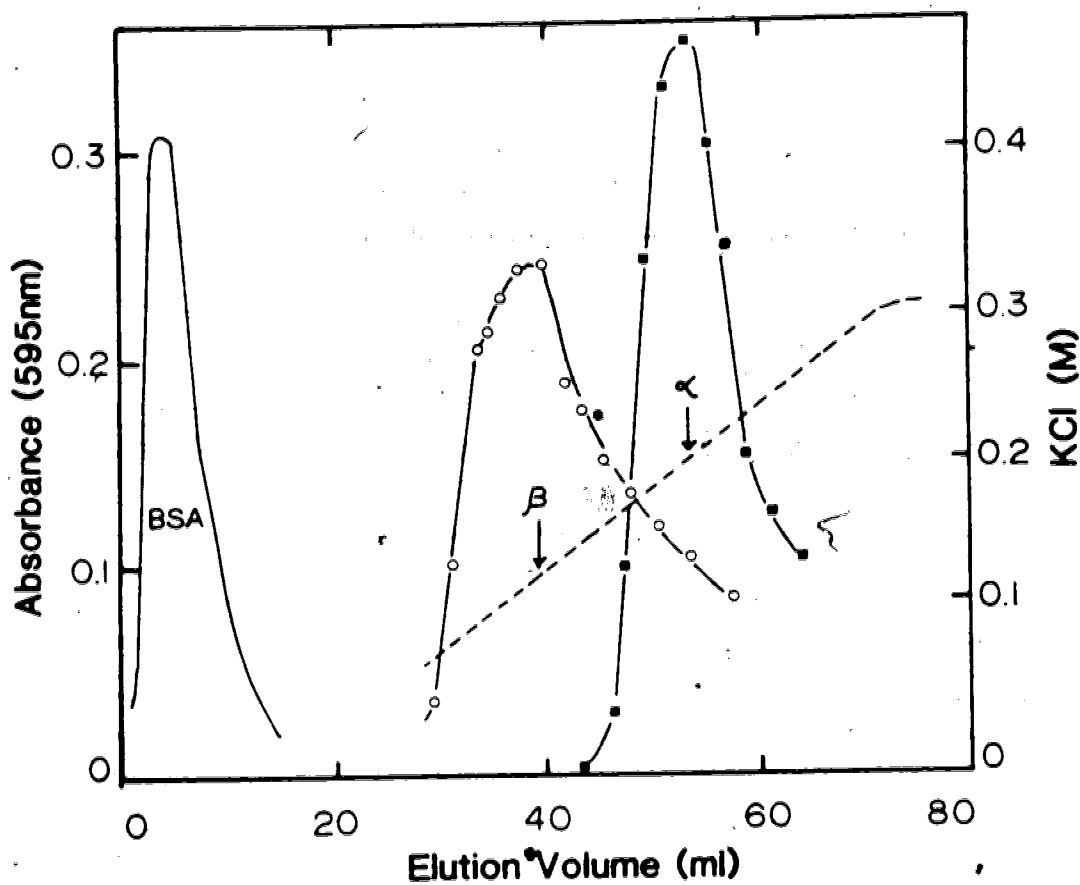


Fig. 17. Elution profiles of bovine serum albumin (BSA) (—),  $\alpha$ -Tm (■—■) and  $\beta$ -Tm (○—○) on a Tn-T Sepharose 4B affinity column. Protein samples of 0.3 mg were dialysed against column buffer (see chapter II) and were applied to the column. After a wash of at least two column volumes, an 80 ml gradient from 0 to 0.5 M KCl (-----) was established. The fraction at the center of each peak (arrows) was used to calculate the ionic strength needed to elute the protein.



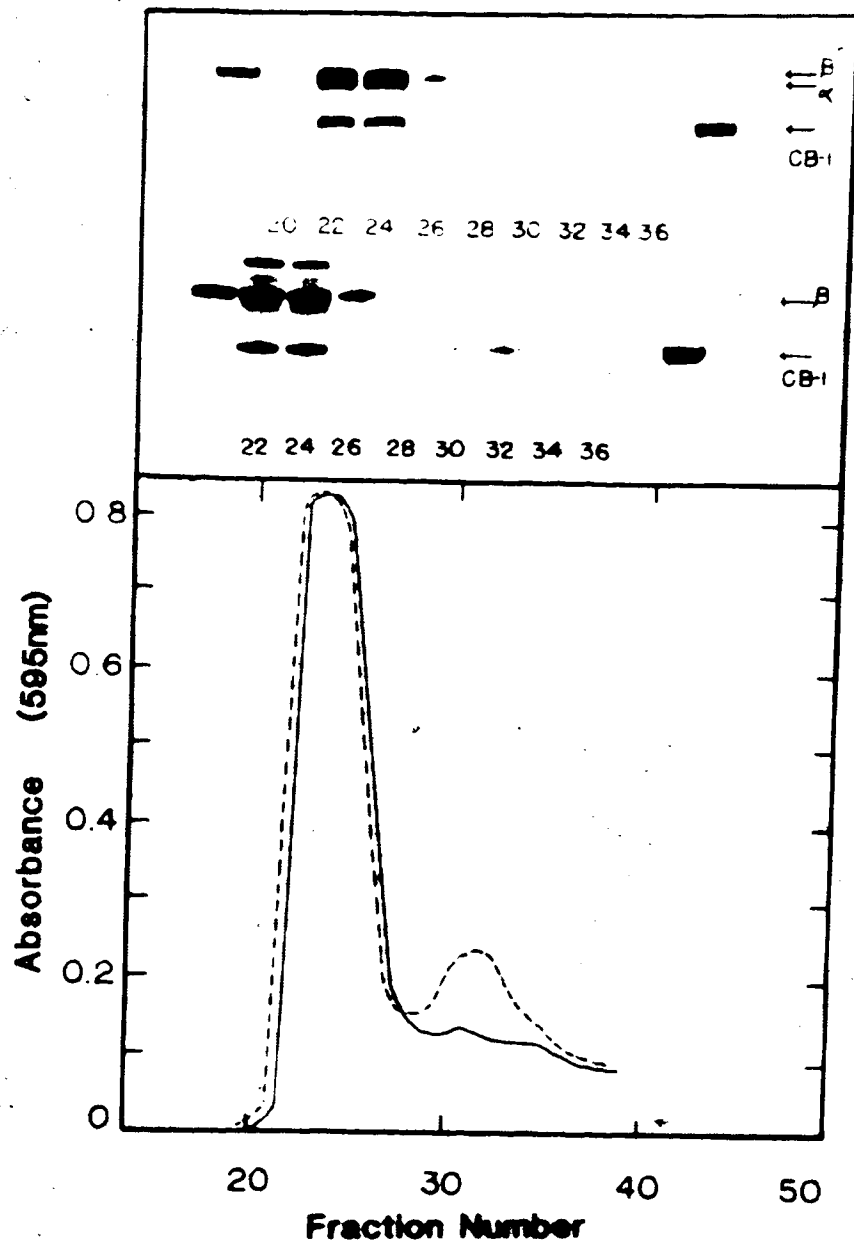


Fig. 18. Gel filtration interaction study of CBI with  $\alpha$ -Tm (—, and gel inset A above) and  $\beta$ -Tm (-----; and gel inset B above) in 0.1 M KCl, 1 mM DTT, 0.01% Na azide and 10 mM imidazole pH 7.0.

In the gels insets, the first unnumbered sample is a  $\beta$ -Tm standard and the last unnumbered sample is a CBI standard. The intermediate samples are selected fractions from the elution profile. Note that Tm dimers sometimes appear as higher molecular weight bands in the tropomyosin-containing fractions. This is more noticeable for  $\beta$ -Tm as it has two Cys residues which makes it harder to reduce.

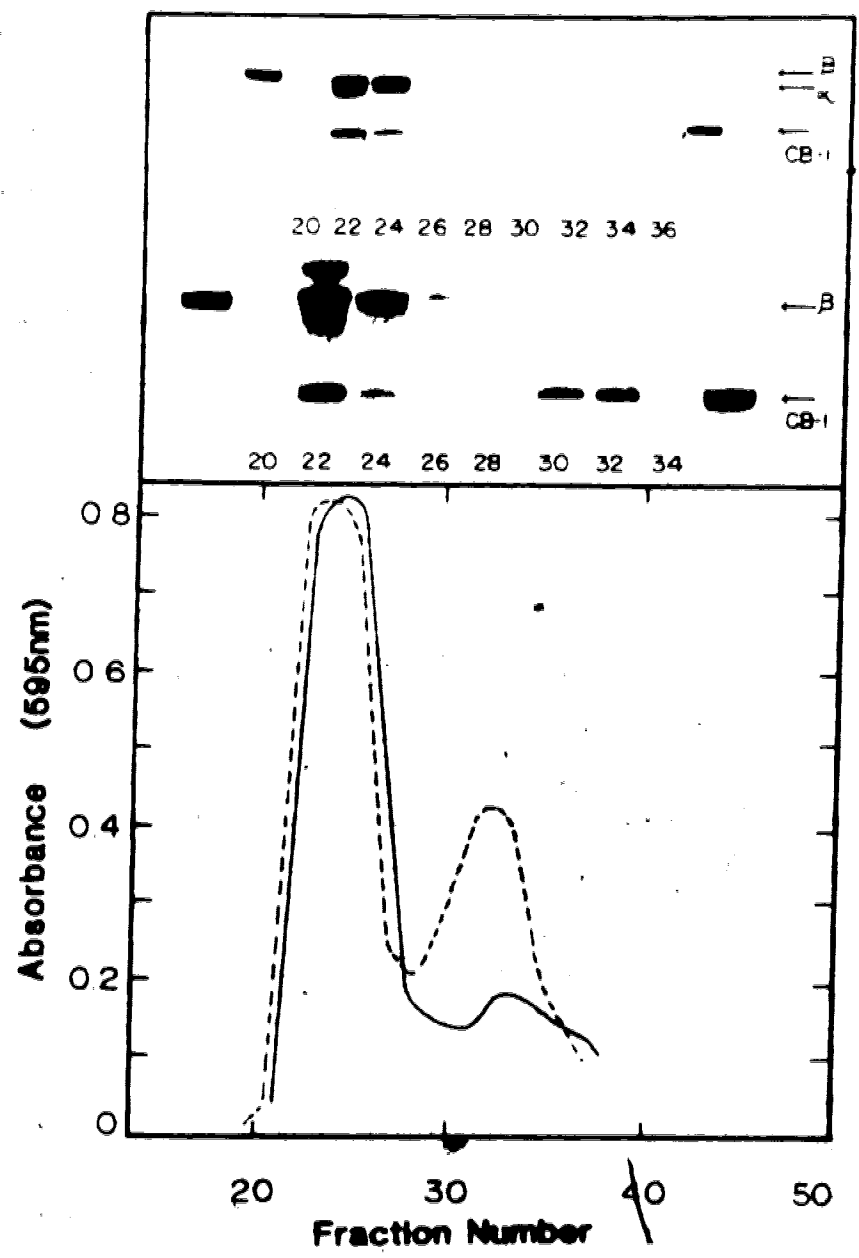


Fig. 19. Gel filtration interaction study of CBI with  $\alpha$ -Tm (—; and gel inset A above) and  $\beta$ -Tm (-----; and gel inset B above) in 0.14 M KCl, 1 mM DTT, 0.01% Na azide and 10 mM imidazole pH 7.0.

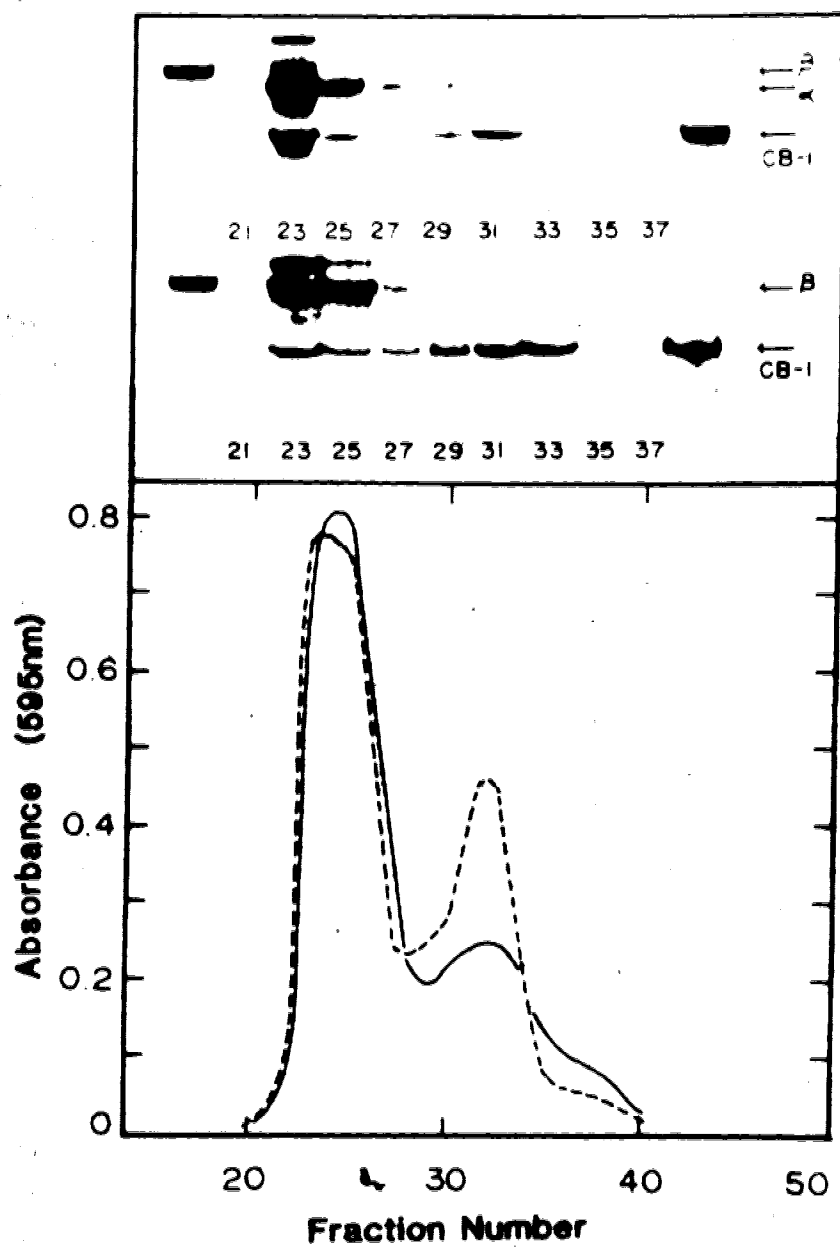


Fig. 20. Gel filtration interaction study of CBI with  $\alpha$ -Tm (—; and gel inset A above) and  $\beta$ -Tm (-----; and gel inset B above) in 0.18 M KCl, 1 mM DTT, 0.01% Na azide and 10 mM imidazole pH 7.0.

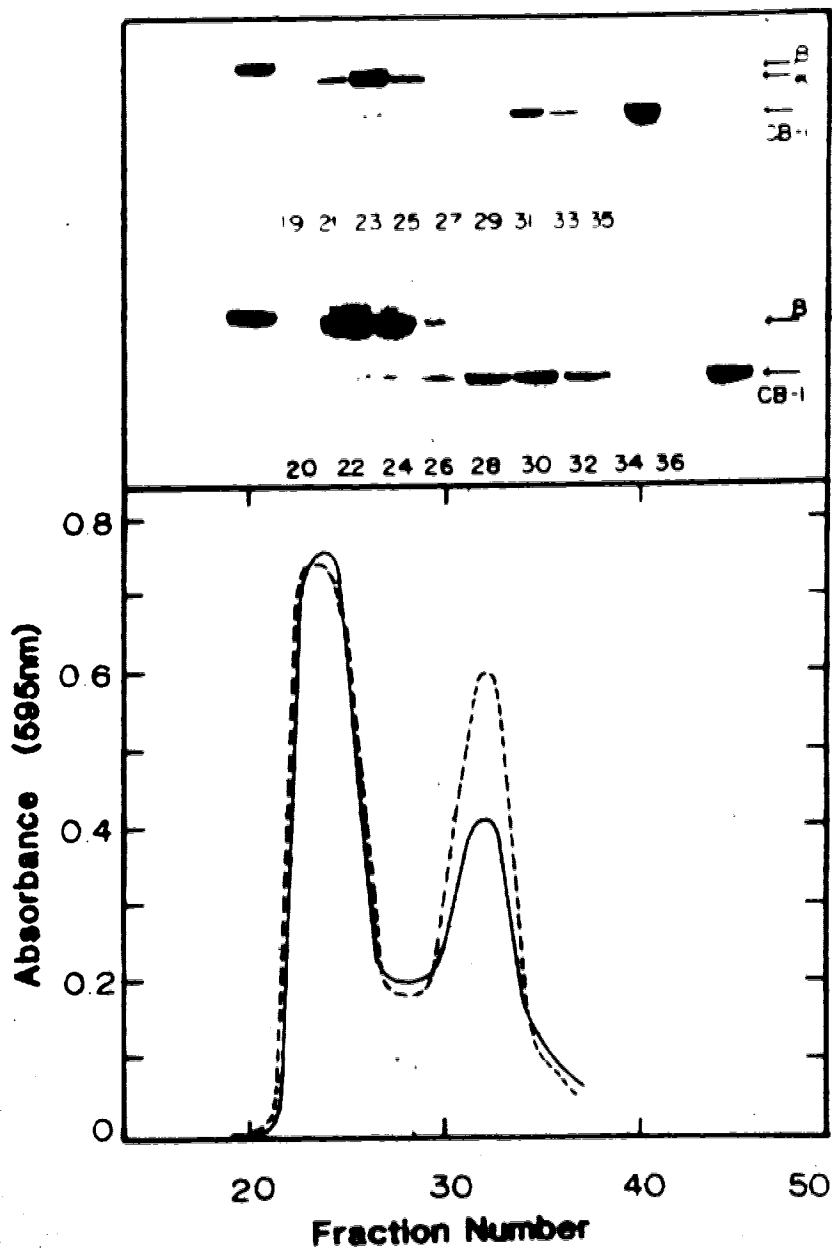


Fig. 21. Gel filtration interaction study of CBI with  $\alpha$ -Tm (—; and gel inset A above) and  $\beta$ -Tm (-----; and gel inset B above) in 0.22 M KCl, 1 mM DTT, 0.01% Na azide and 10 mM imidazole pH 7.0.

mal position in the  $\beta$ -Tm profile (-----), which is indicative of a slightly weaker affinity. At the higher ionic strength of 0.22 M KCl (Fig 20) the proteins ran largely dissociated from one another. The intermediate values (0.14 and 0.18 M KCl) showed the most dramatic differences in CBI binding ability between the two tropomyosins. Clearly, both in the elution profiles and in the gel insets above them, the CBI was seen more tightly associated with the  $\alpha$ -form. Thus the interaction studies by gel filtration agreed well with the previous affinity chromatographic results. In both sets of experiments the dissociation of Tn from  $\beta$ -Tm occurs earlier than for  $\alpha$ -Tm, even though in one case we were dealing with whole troponin and in the other with a fragment of the Tn-T subunit.

### C. TROPOMYOSIN-ACTIN INTERACTIONS

The last set of experiments in this chapter deals with the interaction between tropomyosin and actin. Inspection of the Tm sequence indicates that the 19.7 residue periodicity (Parry, 1974; Stone et al., 1975; McLachlan and Stewart, 1976a) which is repeated 14 times throughout the sequence of  $\alpha$ -Tm is also found in  $\beta$ -Tm, essentially without a change (Mak et al., 1980). These periods can be divided into alternating sets of  $\alpha$  and  $\beta$ -bands which are thought to represent actin binding sites (see introduction). Because most of the amino acid substitutions are chemically similar and because this periodicity is maintained in  $\beta$ -Tm, Mak et al. (1980) predicted that the actin binding properties of both forms of Tm would be similar. Wegner (1980), in studying the interaction of  $\alpha$ -Tm homodimers and  $\alpha,\beta$ -heterodimers with actin filaments by light scattering methods, found that their affinities were very similar. The  $\beta$ -Tm homodimer, however, has not been investigated.

Many factors can affect tropomyosin's ability to bind to F-actin.

Some of these variables (temp, pH, urea) alter the  $\alpha$ -helical content of Tm and lower its binding ability to F-actin (Tanaka, 1972). Increasing ionic strength and the addition of divalent cations also affect this interaction without necessarily affecting the tertiary structure of Tm (see discussion following). For this reason we used these variables in our co-sedimentation experiments.

### 1. Ionic Strength

In these experiments  $^{125}$ Iodine labelled  $\alpha$  or  $\beta$ -tropomyosin was mixed with a standard amount of actin in a binding buffer of 3 mM Tris pH 7.8, 0.1 mM EGTA, 2mM ATP, 2 mM DTT and 30 mM KCl (based on Eaton et al., 1975). For the sake of consistency, binding studies were done on the same day for both Tms using the same actin preparation. As can be seen in Fig. 22, very little binding to actin occurred at the 30 mM KCl concentration. As the ionic strength increased, the extent of association between the two molecules increased, becoming maximal in the physiological range (0.1 to 0.15 M KCl) for both  $\alpha$  and  $\beta$ -Tms. At higher KCl concentrations the affinity of  $\alpha$ -Tm decreased whereas the association between  $\beta$ -Tm and actin only decreased at even higher salt concentrations. The results show that  $\beta$ -Tm has a stronger affinity for F-actin at the higher ionic strengths.

### 2. Magnesium Concentration

Fig. 23 shows the results obtained when the magnesium concentration was varied. The same buffer was used as in the previous experiment, but this time the  $Mg^{2+}$  levels were varied. At about 3.0 mM total  $Mg^{2+}$  a dramatic rise in the amounts of both forms of tropomyosin binding to F-actin occurred, and reached a maximum at about 4.0 mM. Further addition of the divalent cation resulted in no additional increase in association. In order to interpret these results it must be remembered that there was 2mM

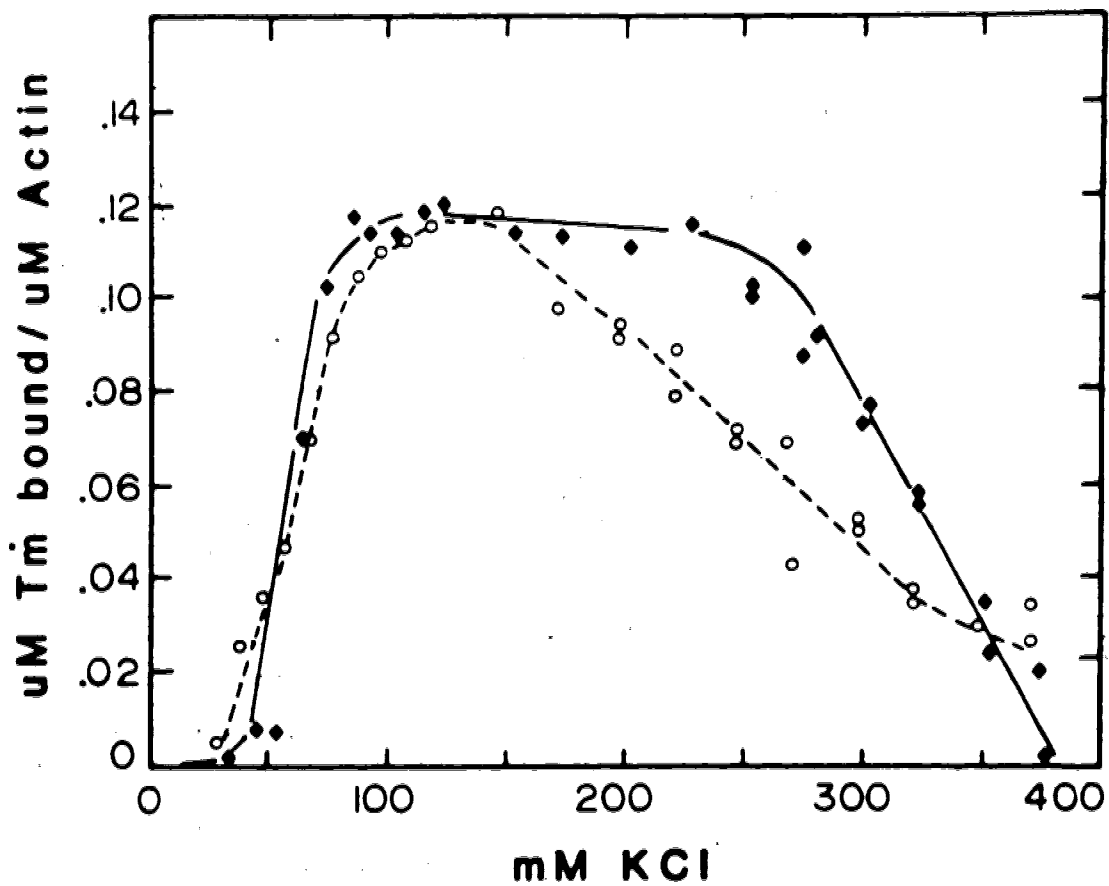


Fig. 22. Effect of KCl concentration on the binding of skeletal  $\alpha$ -Tm (O) and  $\beta$ -Tm (◆) to F-actin. Each tube contained 10  $\mu$ M actin and 2.8  $\mu$ M Tm (2/7 ratio Tm to actin) in a 1 ml volume of 2.0 mM DTT, 2 mM ATP, 0.1 mM EGTA and 2 mM Tris pH 7.8. The KCl concentration was varied from 30 to 400 mM.

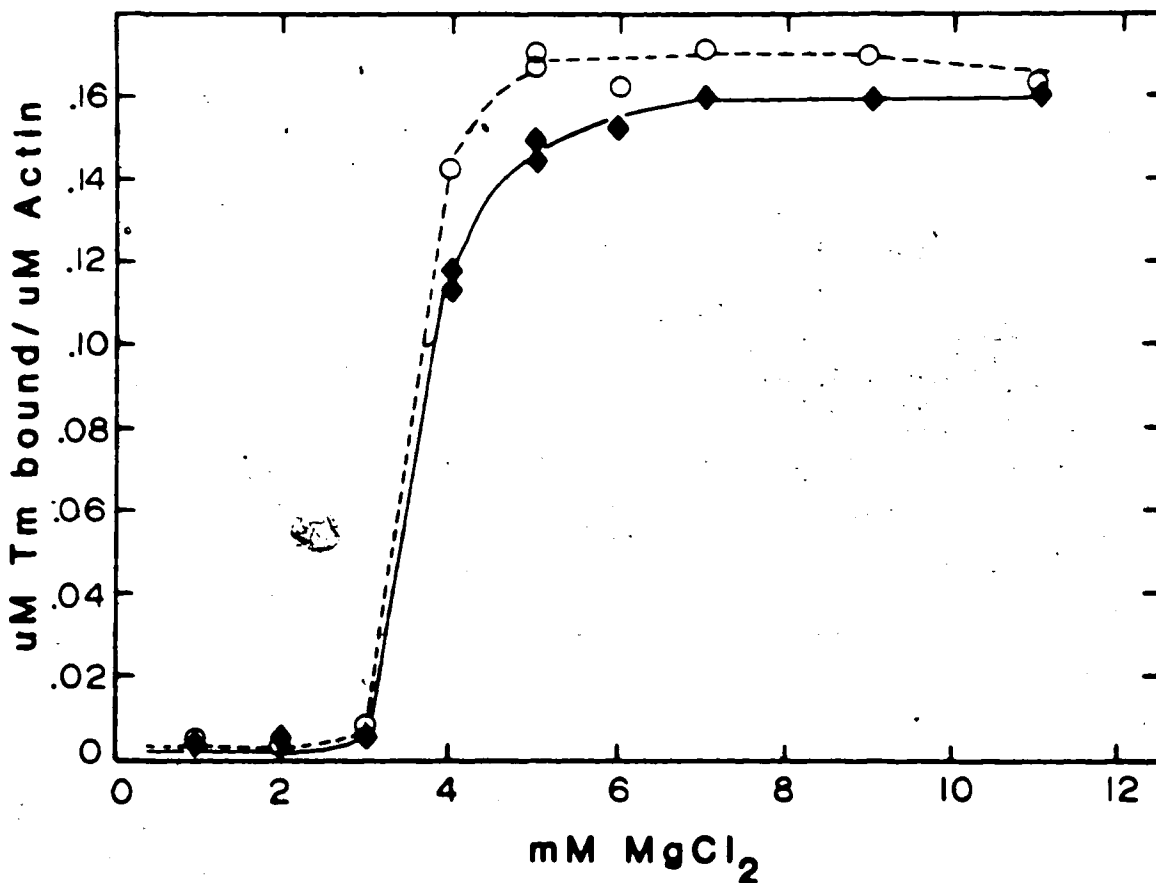


Fig. 23. Effect of magnesium concentration on the binding of skeletal  $\alpha$ -Tm (O) and  $\beta$ -Tm (◆) to F-actin. Conditions were the same as in Fig. 22, only the KCl concentration was kept constant at 30 mM and the magnesium ion concentration was varied from 0 to 12 mM. (Note, the "free magnesium concentration" starts after the 2 mM mark, since before that point all the added ions are complexed by ATP in the binding buffer).



ATP in the binding buffer and that the  $Mg^{2+}$  ions which were added first were bound in a Mg-ATP complex. Only after all of the ATP had been "titrated" could excess amounts of magnesium assist in the protein-protein interactions. This occurred very soon afterwards; in the range of 1 to 1.5 mM "free" magnesium ions.

#### D. DISCUSSION

The technique of viscosity has been used in this section in order to measure the self-association of the tropomyosin molecules as well as the troponin-tropomyosin interactions. In both sets of experiments no differences were seen between the two forms of tropomyosin in these respects. Head to tail polymerization falls off at the higher salt concentrations, as does the interaction with Tn, indicating that both processes are dependent on ionic interactions between the molecules. The fact that stoichiometric Tn/Tm association is seen in the ultracentrifuge even at 1.0 M KCl levels may be due to the fact that side by side aggregation (and perhaps a small amount of head to tail association) can still occur (Hartshorne and Mueller, 1967; Greaser *et al.*, 1972), whereas the long asymmetrical particles seen with electron microscopy at lower ionic strengths (Yamamoto and Maruyama, 1973) would be abolished. It is these aggregated forms that contribute most to viscosity measurements. Studies at still higher ionic levels would be necessary in order to visualize differences with the techniques of viscosity, and at these concentrations it may be difficult to get as accurate data as with the lower salt concentrations.

Our studies with affinity chromatography on a Tn-Sepharose 4B column provided some interesting results. Here it was clearly seen that the ability for  $\beta$ -Tm to bind to the column was considerably less than

for  $\alpha$ -Tm. Both tropomyosins eluted well below 0.25 M KCl (in the range where viscosity measurements had indicated that no differences were apparent. The reason for this discrepancy could be due to the fact that chemical coupling of troponin to the column could sterically alter the binding sites for tropomyosin in such a manner that the protein-protein interactions are weakened. Still, the specificity of the affinity column is retained for tropomyosin since the BSA control came out in the void volume. Taken quantitatively, the results show us that  $\beta$ -Tm has a lower affinity for troponin relative to  $\alpha$ -Tm.

Gel filtration studies using CBI confirmed the affinity chromatographic results. The CBI fragment of Tn-T has a strong affinity for both Tms at the lower ionic strengths but its associations are reduced at the higher salt levels. The range of KCl which is needed to observe this dissociation (0.1 to 0.2 M) correlates well with the results from the Tn-Sephrose 4B column (0.12 to 0.2 M), but since we were using a fragment, we cannot predict if whole troponin would give similar results. Nevertheless, a definite difference between  $\alpha$  and  $\beta$ -Tms does exist with respect to troponin binding ability. The  $\beta$ -Tm association is weaker.

It is of interest to attempt to relate this observation to the known differences in the amino acid sequences of the two forms of tropomyosin. McLachlan and Stewart (1976b) suggested that residues 197 to 217 may be the region where Tn-T binds on tropomyosin, since this segment of the sequence has more irregularities than any other. Inspection of the  $\alpha$  and  $\beta$ -sequences reveals that there are only three amino acid substitutions here; Thr<sub>199</sub> to Ile, Glu<sub>212</sub> to Asp and Gln<sub>216</sub> to Thr. It is hard to imagine only three conservative residue changes giving rise to the rather large differences which were observed in the previous experiments. As

well, a variety of studies on the nature of the Tn-T molecule (Pearlstone et al., 1976; Pearlstone and Smillie, 1977; 1980) suggest that Tn-T is an open asymmetric structure (summarized in Fig. 24) capable of spanning an extensive region of Tm. Immuno-electron microscopic observations by Ohtsuki (1975, 1979) confirm these results. Antibodies raised against Tn-T and its chymotryptic fragments T<sub>1</sub> and T<sub>2</sub> bind over a broader region of the thin filament (10 nm) than do the Tn-C and Tn-I antibodies. The work of Mak and Smillie (1981b) provides evidence that CBI binds near the region of Tm overlap, since tyrosines 261 and 267 were largely protected from labelling with <sup>125</sup>Iodine in the presence of this fragment. As well, non-polymerizable tropomyosin (NPTm) which has its 11 COOH-terminal residues removed through carboxypeptidase A digestion, has a reduced affinity for CBI (Mak and Smillie, 1981a). There is much evidence, then to suggest a more extensive binding region for troponin.

Mak and Smillie (1981b) have proposed that the two-site binding of tropomyosin on the thin filament (Potter and Gergely, 1973; Hitchcock et al., 1975; Margossian and Cohen, 1973) may be interpreted in terms of a binding of the CB2 region of Tn-T (residues 71 to 151) close to or at the head to tail overlap region of the tropomyosin molecules. A second binding site may exist in the region of Cys<sub>190</sub> of Tm and the COOH-terminal region of Tn-T (residues 197 to 250) (Pearlstone and Smillie, 1981). Since CBI of Tn-T mimics whole troponin in its differential binding to  $\alpha$  and  $\beta$ -tropomyosins, it would seem that these differences are more likely attributable to differences in the amino acid sequence of the two forms in the region of residues 258 to 284 of the tropomyosin molecule. In comparing the two sequences, five such differences are known:

## STRUCTURAL FEATURES OF TROPONIN-T

- 259 amino acid residues.
- 50% are charged at pH 7.0.
- no significant stretches of non-polar residues.
- $\alpha$ -helical content (35%) largely localized in fragment CB2 (residues 71-151) which is 80%  $\alpha$ -helical and stable over pH 3.3 - 9.1 and 0.1 - 1.1 M KCl.
- this stability inconsistent with CB2 existing as single extended  $\alpha$ -helix in isolation.

Conclusion: Tn-T probably exists as open asymmetric molecule with helical CB2 folded back on itself.

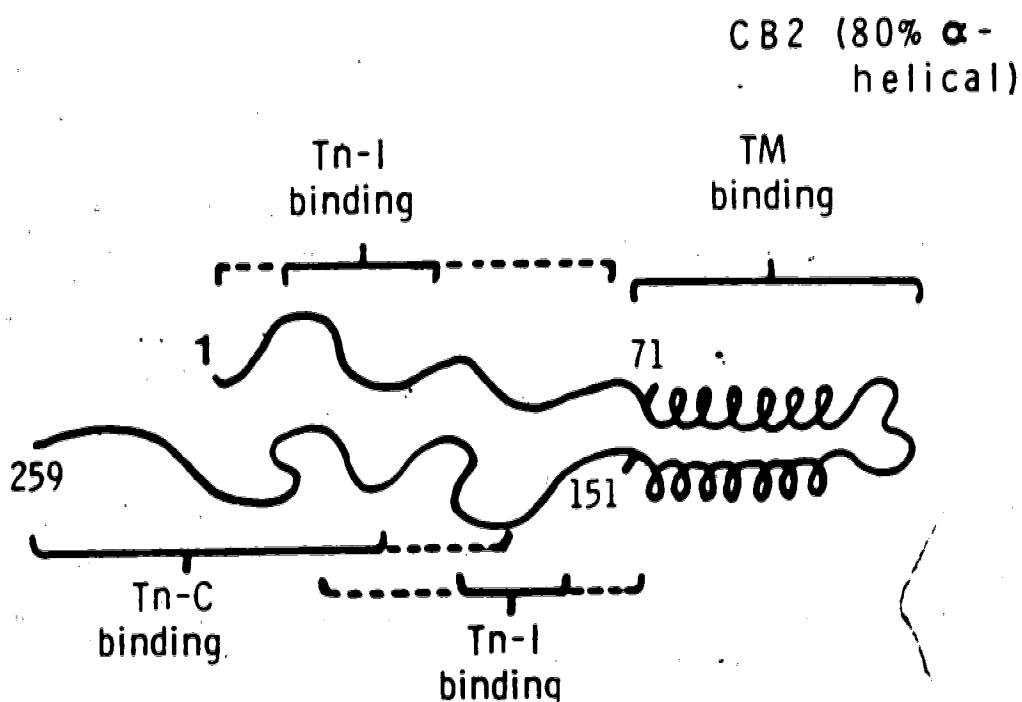


Fig. 24. The structural features of Tn-T. (Summarized from Pearlstone et al., 1976; Pearlstone and Smillie, 1977; 1980).

<u>Residue #</u>	<u><math>\alpha</math>-Tm</u>	<u><math>\beta</math>-Tm</u>
260	Leu	Val
265	Leu	Met
276	His	Asn
281	Met	Ile
284	Ile	Leu

Four of these are highly conserved and only the substitution His<sub>276</sub> by Asn might be expected to alter significantly the binding properties of Tm to the CBI fragment of Tn-T. However, the possibility that other substitutions more distant from the COOH-terminal region may have an effect on the binding properties of CBI cannot be ruled out. Such substitutions, remote from the direct binding site for CBI, could lead to subtle changes in the stability and conformation of the coiled-coil structure of the Tm molecule which could be transmitted to the CBI binding region and lead to changes in its affinity for this portion of the Tn-T molecule. That this is possible is indicated by the fact that  $\beta$ , $\beta$  dimers of Tm are less stable to heat denaturation and that conformational transitions may be transmitted for long distances along the coiled-coil structure (Edwards and Sykes, 1980).

Actin binding in this chapter was studied with the technique of co-sedimentation. Tropomyosin and actin were mixed together and then sedimented for 1.5 h at 100,000 Xg in an ultracentrifuge. At this speed the actin filaments pellet out, carrying with them any associated proteins. Control experiments without F-actin indicated that less than 5% of the Tm came down under these experimental conditions. Because Tm was radioactively labelled, an accurate estimate of how much sedimented could be obtained (see methods), eliminating the uncertainties involved with SDS PAGE analysis of pellets and supernatants.

We chose to study actin/Tm binding under two sets of conditions;

increasing ionic strength and increasing  $Mg^{2+}$  concentrations. Neither of these has been reported to alter the helix content of Tm (decreased helix content decreases F-actin binding ability). Binding of both  $\alpha$  and  $\beta$ -Tms increases as the ionic strength increases, becoming maximal at 0.1 M KCl (Maruyama, 1964; Tanaka, 1972; Eaton et al., 1975). KCl probably functions by changing the apparent charge on protein molecules, thus modifying their interactions. It was gratifying that maximal associations occurred in the physiological range of ionic strength. Shortly after this region, however, further increase of salt concentration led to the appearance of differences between the  $\alpha$  and  $\beta$ -tropomyosins. The  $\beta$ -form was seen to have a stronger affinity for F-actin at the higher ionic strengths. Eventually, though, it too lost its ability to bind F-actin.

Co-sedimentation studies under conditions where the  $Mg^{2+}$  ion concentration was increased stressed their role in Tm/actin associations. Even under conditions where very little binding was seen (30 mM KCl), 1-2 mM "free"  $Mg^{2+}$  can induce tropomyosin to bind maximally to F-actin (Tanaka, 1972; Eaton et al., 1975; Wegner, 1979; Yang et al., 1979a,b). How  $Mg^{2+}$  ions accomplish this feat is not completely clear. They could act as a salt bridge between neighbouring negatively charged residues thus stabilizing their normally unfavourable interactions, or they could stabilize the F-actin polymer (Tanaka, 1972). At any rate, no differences between the two forms of tropomyosin were detected by varying the magnesium concentration.

How these results relate to the amino acid sequences is presently unclear. Since most of the 30 amino acid substitutions are chemically conservative, the putative actin binding sites are not altered much (Mak et al., 1980). Nevertheless the experiments provide evidence that  $\beta$ -Tm

(at least at the higher ionic strengths) has a stronger affinity for F-actin.

It is very difficult to judge the physiological relevance of these results, given the limitations of the experiments themselves and the complex nature of the in vivo situation. It is tempting to speculate, however, that  $\beta$ -Tm is more of a structural protein than a regulatory one. Because  $\beta$ -Tm has a relatively stronger affinity for F-actin and a weaker one for troponin, it may be less effective in transmitting the  $\text{Ca}^{2+}$  induced signals from troponin through to the F-actin monomers (or, perhaps in rolling closer into the actin grooves upon activation). In embryonic muscle the need for quick responses to external stimuli is minimal. Here the increased amounts of  $\beta$ -Tm may serve a more functional role in organizing the newly developing thin filaments.

Certainly this speculative theory cannot be easily checked experimentally. However it is possible to compare both tropomyosins with respect to their abilities to function as "regulatory" proteins by using a reconstituted actomyosin ATPase system. This will be dealt with in the next chapter.

## CHAPTER IV

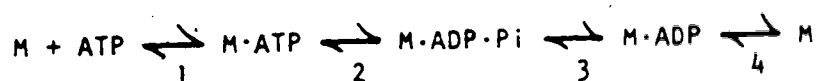
ACTOMYOSIN ATPASE STUDIES WITH  $\alpha$  AND  $\beta$ -TROPOMYOSINS

## A. INTRODUCTORY REMARKS

1. The Actomyosin ATPase

It is now generally accepted that muscle contraction is a result of the relative sliding of the thick filaments past the thin filaments and that this is accomplished by cyclic interactions of the myosin heads with the actin monomers (Huxley, 1969). The hydrolysis of ATP by myosin supplies the energy needed for this movement. This ATPase activity has been a great asset in muscle research since it provides quantitative information of the events occurring during contraction.

The scheme below illustrates the essential features of the myosin ATPase:



where M refers either to whole myosin or to its proteolytic fragments HMM (heavy meromyosin) and S-1 (the subfragment-1 of HMM). All three have the ability to cleave ATP but the fragments are soluble at the lower ionic strengths where myosin is not (Lowey *et al.*, 1969).

In step 1 above, myosin (S-1) and ATP associate. There is a very large drop in free energy here and the binding is essentially irreversible. In step 2, ATP is hydrolysed to ADP·Pi (the initial Pi burst) which occurs at a rate of 150-200 s<sup>-1</sup>. Step 3 is rate limiting (V<sub>max</sub>) above 5°C. whereas step 4, the release of ADP is rate limiting below 5°C. Product release (step 3 for our purposes) is very slow (0.05 s<sup>-1</sup>) under physiological conditions (0.15 M KCl, 5-10 mM MgCl<sub>2</sub>, pH 7.0) but increases with increasing



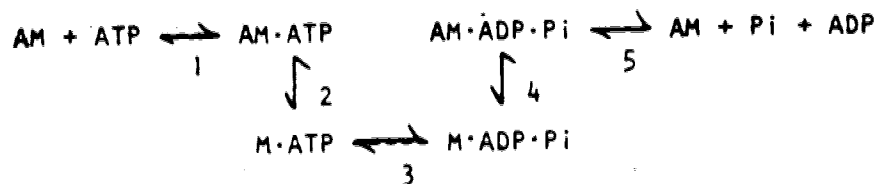
pH and ionic strength. The  $K^+$ -EDTA ATPase assay mentioned in chapter 11 (which is done at 0.6 M KCl, 5 mM EDTA, pH 7.8) will give a turnover number for step 3 of between 6-13  $s^{-1}$ . It is useful in providing an indication of myosin purity during preparative procedures since at this high ionic strength myosin is soluble and interactions with other proteins (in the presence of ATP) are largely abolished. For a more complete analysis of the myosin ATPase the articles by Taylor (1979) and Shrivvers and Sykes (1981) are recommended reading.

## 2. The actomyosin ATPase

It has long been known that the addition of actin to myosin at physiological ionic strengths would activate the ATPase. Eisenberg and Moos (1968;1970) performed the initial steady state kinetic studies which were the foundations of all future work. They showed that the actin-myosin interactions follow the rules of simple kinetics. By keeping the S-1 concentration constant and increasing actin, there was a hyperbolic increase in ATPase activity. Double reciprocal plots yielded information on  $V_{max}$  (the maximum ATPase at saturating actin) and  $K_m$  (the amount of actin needed to give  $1/2 V_{max}$ ). Actin was seen to increase the rate of product release from myosin 200 fold ( $10 s^{-1}$ , pH 8.0, 20°C, low ionic strength). The  $K_m$ , which is also a measure of the affinity of myosin for actin, was shown to increase with increasing ionic strength whereas  $V_{max}$  was affected to a much smaller degree. Overall, actin was seen to greatly accelerate product release (step 3 of the myosin ATPase).

Pre-steady state kinetic studies of Lynn and Taylor (1971) shed more light on the picture. They studied the kinetics of actomyosin dissociation in the presence of ATP and found that at stoichiometric levels of actin and S-1, this rate was faster ( $500 - 1000 s^{-1}$ ) than the subsequent

Pi burst ( $150 \text{ s}^{-1}$ ). Combining this feature with the other relevant features of the ATPase they came up with the following model:



The binding of ATP to actomyosin resulted in a large drop in free energy and was essentially irreversible. Step 2, the dissociation step, occurs faster than step 3, the initial Pi burst. Now the myosin-products complex is seen to associate again with actin ( $K_m$ , ionic strength dependent) and product release follows ( $V_{max}$ ). The attractive feature of this model is that it depicts one cycle of dissociation and reassociation for each ATP hydrolysed, which was also postulated to occur in vivo during contraction.

The Lymn and Taylor model predicts that at  $V_{max}$  most of the myosin will be in the  $\text{AM}\cdot\text{ADP}\cdot\text{Pi}$  form (since product release is rate limiting). Eisenberg and co-workers (1972a,b) tested this prediction under conditions where actin would approach the effective concentration in muscle<sup>1</sup>. Scanning ultracentrifugation studies at  $5^\circ\text{C}$  showed that most of the S-1 was dissociated from actin under  $V_{max}$  conditions, contradicting Lymn and Taylor's postulation. Eisenberg explained his results by adding another step in the model above. The myosin, after the Pi burst, was considered to be in a non-refractory state; it could not bind to actin until it had undergone a rate-limiting conformational change to a "refractory state" (the actin binding form).

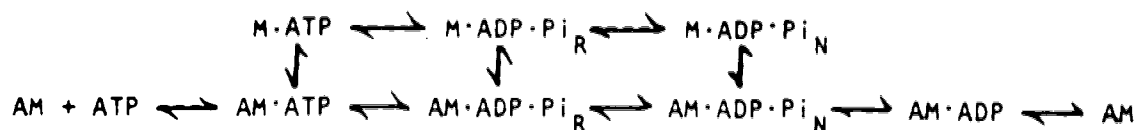
Eisenberg's refractory state model predicts that at  $V_{max}$  all the

Even though in muscle the ratio of actin to myosin is approximately 4 to 1, the fact that the proteins are oriented in the filaments means  $V_{max}$  conditions are approached at lower ratios than in solution, where the association process is random.

S-1 should be dissociated from actin. The experiments, however, had been done at 5°C where the AM·ATP  $\rightleftharpoons$  M·ATP step (which is reversible) was pushed far to the right (M·ATP form). However at higher temperatures Marston (1978) and Wagner and Weeds (1979) found considerable association of actin and myosin in the presence of ATP. Stein et al., (1979) using stopped flow absorbance, extended these observations. When actin and myosin were mixed together with ATP, they observed that a rapid equilibrium was set up between the components. At low actin concentrations, AM association in the presence of ATP was small, but increased hyperbolically with increasing actin levels. In this way a  $K_m$  of binding could be obtained. There was a 4 fold difference in this binding constant (M·ADP·Pi to actin) and the  $K_m$  for the ATPase activity, suggesting that the refractory state was still valid, and was the slowest step in the cycle.

If ATP hydrolysis can only occur when S-1 is off the actin, then these results predict a decrease in activity when actin levels are high (since a considerable amount of S-1 will be complexed with actin). Stein et al., (1979) found no inhibition in the ATPase at the high actin levels (greater than 200  $\mu$ M) and so concluded that ATP hydrolysis could occur in the actomyosin complex. In other words, dissociation of the myosin head is not necessary to hydrolyse ATP.

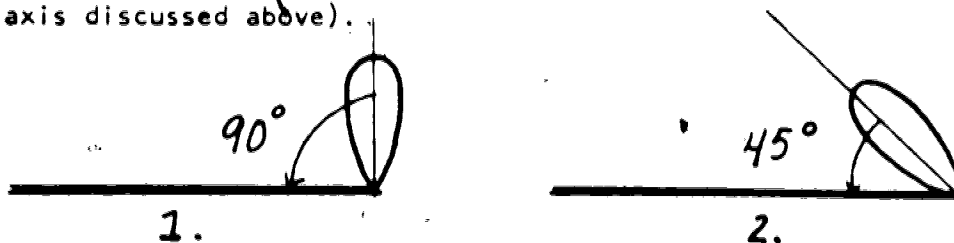
The model below is based on this biochemical evidence and is known as the modified refractory state model. It is presented in a simplified version below:



In summary then, the actomyosin complex binds ATP and dissociates (when the actin concentration is low) or stays associated (when actin concentrations are high). In either case, hydrolysis of ATP occurs (initial Pi burst) followed by a rate limiting transition to a form which associates strongly with actin. Finally, product release occurs.

### 3. Cross Bridge Models

Cross bridge models are highly theoretical and beyond the scope of this thesis. However, a few major points need to be brought forward here in order to clarify future discussions. Myosin heads are thought to bind to actin in two major states; with a  $90^\circ$  angle and with a  $45^\circ$  angle (relative to the long axis of the myosin filament). These values were derived from diffraction patterns of resting muscle and muscles in rigor. Huxley and Brown (1967) determined that in relaxed muscle the myosin heads do not touch the actin filaments and that they extend perpendicular with respect to the long axis of the thick filaments ( $90^\circ$ ). In rigor muscle the diffraction pattern (Moore et al., 1970) is altered. The shifts in intensity have been interpreted such that the myosin heads bind actin and have another angle of attachment ( $45^\circ$  with respect to the long axis discussed above).



State 1 is thought to occur with  $M \cdot ATP$  and  $M \cdot ADP \cdot Pi$  whereas state 2 is thought to occur with  $M \cdot ADP$  and nucleotide free myosin (see also Fig. 5 in the Introduction). The transition from state 1 to state 2 represents the "power stroke".

Eisenberg and Green (1980) have proposed a current model of cross-

bridge action. Cross bridges are considered to be elastic structures which can bind over a wide range of angles ( $45^\circ$  and  $90^\circ$  are preferred angles). Certain binding angles ( $90^\circ$ ) are assumed to be in rapid equilibrium with their unattached states, and all transitions in the cross-bridge cycle are considered to be reversible.

#### 4. Mechanisms of Regulation by Tropomyosin-Troponin

The interactions between actin and myosin are modified in the presence of the regulatory proteins, tropomyosin and troponin (Tm·Tn). The steric blocking model suggests that Tm physically blocks myosin binding sites in relaxed muscle, whereas in the presence of calcium it rotates towards the center of the F-actin grooves and allows the myosin intermediates to bind. Under some conditions, however, ATPase activity occurs even in the absence of  $\text{Ca}^{2+}$ . Bremel et al., (1972) showed that at low ATP levels, S-1-ADP could bind tightly to regulated actin as "rigor complexes" thus pushing the relatively rigid tropomyosin molecules into the F-actin grooves and opening binding sites for the remaining S-1-ADP·Pi intermediates. This was not incompatible with the steric blocking model since S-1-ADP was considered to successfully "compete" with tropomyosin for actin binding sites.

Greene and Eisenberg (1980) extended these observations by showing that S-1-ADP bound more weakly to regulated F-actin (less than  $10^3 \text{ M}^{-1}$ ) than to unregulated F-actin ( $2 \times 10^5 \text{ M}^{-1}$ ). However, once the S-1-ADP had gained access to the regulated filaments (higher levels of saturation), the S-1-ADP bound 3 times more strongly ( $7 \times 10^5 \text{ M}^{-1}$ ). This did not disagree with the steric blocking model either, except that the same phenomenon occurred (with essentially the same binding constants) for filaments in the presence of calcium, the only difference being

that the cooperative transition between the weak and strong binding forms occurred sooner. In the presence of  $\text{Ca}^{2+}$  the steric blocking model would predict that S-1-ADP should have a similar affinity as it has to unregulated actin filaments. If regulated actin can exist in two forms (weak or strong with  $\text{Ca}^{2+}$  and S-1-ADP being allosteric effectors between the two states) then the binding of the other myosin intermediates would be expected to follow a similar pattern as S-1-ADP.

Recently Chalovich et al. (1981) tested the affinity of S-1-ATP and S-1-ADP·Pi to regulated actin filaments using stopped flow absorbance measurements. Their results surprisingly showed that the Tm·Tn complex had very little effect on the binding of these intermediates. Both in the presence and absence of calcium this value was  $1.3 \times 10^4 \text{ M}^{-1}$ , even when the ATPase activity was 96% inhibited! Since inhibition of binding for these 90° cross bridge states does not occur whereas the 45° states are affected, they speculate that Tm·Tn must somehow inhibit product release (the 90° to 45° transition power stroke). Since the 90° cross bridge intermediates can interact with relaxed filaments, they were postulated to be in rapid equilibrium with their unattached states. This would account for the lack of stiffness in relaxed muscles.

One final feature of regulated filaments must still be explained, and that is their ability to increase the ATP hydrolysis of myosin above the level of unregulated actin filaments. This is called "potentiation", and it occurs in the presence of rigor complexes and also at higher ratios of S-1/actin (Bremel et al., 1972). It is difficult to see how potentiation can be explained in terms of the steric blocking model. Once Tm has moved to the groove (uncovering all the myosin binding sites on the F-actin monomers) how can it give rise to the superactive state? Can

it be that Tm can induce conformational changes in the F-actin monomers, thus increasing their affinity for the myosin intermediates?

Evidence does exist that actin plays more than just a passive role in the regulatory processes. Poo and Hartshorne (1976) have reported that glutaraldehyde cross-linked actin filaments are "frozen" in the active state and are no longer subject to inhibition by Tm·Tn, even though their binding is not impaired. As well, Yang et al. (1977;1979a, b) working on Acanthamoeba and skeletal actin co-polymers have shown that tropomyosin seems to affect each type of actin monomer differently.

#### B. THE ACTIN-ACTIVATED MYOSIN SUBFRAGMENT-1 ~~ATPASE~~ SYSTEM

Since all of the work in this chapter will deal with the actin-activated Mg<sup>2+</sup>-dependent S-1 ATPase (actin-S-1 ATPase for short) a few of the controls and precautions used in the following experiments will be documented here.

ATP hydrolysis can be conveniently followed using a pH electrode (pH stat method). Protons liberated from the ATPase reaction are titrated with a known amount of base and this quantity is registered directly with a recording device, thus giving quantitative estimates of the rate. For our purposes the pH stat method was especially useful as we wanted to examine the effects of the regulatory proteins on the actin-S-1 ATPase immediately upon their addition, one at a time, both in the absence and presence of calcium. The phosphate determination method (which involves taking aliquots of a reaction mixture at timed intervals to estimate the release of inorganic phosphate) would have been much more cumbersome and also not quick enough to measure results of a more transient nature.

The use of a pH electrode does restrict the experimental design

somewhat. Extremely high concentrations of proteins and the presence of DTT tend to interfere with its performance and so must be avoided. As well, the electrode cannot distinguish between protons released due to ATP hydrolysis and those of another origin. Thus it is important to have all proteins, buffers and the ATP stock solution at the proper pH. Even under optimal conditions a small amount of electrode drift occurs, so in situations where the hydrolysis rate is low, the accuracy of the pH stat method falls off.

As previously mentioned, the association between actin and myosin (hence the rate of the ATPase) is ionic strength dependent. At physiological KCl concentrations (0.1 to 0.15 M KCl) this interaction is very weak and the rate of ATP hydrolysis is difficult to detect (with any margin of accuracy) with the electrode. For this reason our experiments were done at lower ionic strengths (4 mM and 30 mM KCl).

Enzyme kinetics are usually done at saturating levels of substrate. In our case actin may be considered kinetically equivalent to a substrate of myosin S-1. Preliminary experiments at 4 mM KCl, 5 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 2 mM Tris pH 7.8, 2 mM ATP and 2 mM DTT indicated that even at these low ionic strengths we would still need 100  $\mu$ M actin to attain V<sub>max</sub>. At this concentration, actin is extremely viscous and tends to interfere with the performance of the electrode. As a compromise we chose more stoichiometric ratios of S-1 to actin, but found it necessary to do a number of control experiments in order to determine the effect of dilution on the rate of ATP hydrolysis (dilution alters the relative concentration of the proteins in the assay mixture). Volumes up to 0.2 ml (of buffer of the same ionic strength) per 2 ml assay, decreased the ATPase activity



by less than 5%. For this reason subsequent additions to our assay mixture were kept to within this limit. As well, 0.1 mM free calcium had little (less than 5%) effect on the observed rates, although at higher levels (1.0 mM) significant inhibition (40%) of the ATPase was seen.

Once the controls were set up, the assay conditions were tested. S-1 and actin concentrations were chosen and were kept constant between sets of assays. For each assay the initial rate of ATP hydrolysis for these two proteins alone was taken in the presence of ATP (100%) and then the regulatory proteins were added one at a time (tropomyosin first, then troponin) in order to determine their effects on this rate. Calcium was the final addition and it provided a measure of the "calcium sensitive release". At 4 mM KCl, however, we saw very little release of inhibition in the presence of  $\text{Ca}^{2+}$ . When we tried the assay at 30 mM KCl with the same proteins, more "physiological" results were obtained; good inhibition by the Tm-Tn regulatory proteins (up to 65%) and good release of inhibition by calcium. Our conditions, then were the following: 30 mM KCl, 5 mM  $\text{MgCl}_2$ , 2 mM DTT (used only to pre-reduce the proteins but not in the assay itself), 0.1 mM EGTA, 2 mM disodium ATP and 2 mM Tris pH 7.8. These conditions are similar to those of our binding buffer (chapter 3) except that magnesium is added in order to ensure that the tropomyosin will bind to the actin filaments at 30 mM KCl.

ATP hydrolysis in an acto-S-1 system is linear over a wide range of ATP concentrations (Bremel et al., 1972) thus product inhibition is not a problem. However, when ATP levels fall below  $10^{-5}$  M, S-1-ADP rigor complexes may become abundant and potentiate ATP hydrolysis in a short burst before the remaining substrate is exhausted. This phe-

numinum is seen with regulated filaments only). In order to avoid formation of rigor complexes, our measurements were performed before 25% of the ATP had been hydrolysed (1.5 mM ATP left). This was occasionally checked by letting the titration go to completion.

All experiments in this chapter were performed using S-1 of high specific activity; 13-14  $s^{-1}$  in the  $K^+$ -EDTA system. Wagner and Weeds (1979) had shown that the different isomers of S-1 (S-1-A1 or S-1-A2) have slightly different properties in the actin-activated S-1 ATPase system at low ionic strength (6 mM KCl), but that these differences were less pronounced at the higher salt concentrations (26-46 mM). For this reason we pooled both isomers together (Fig. 11) to form a population of S-1 which was representative of the rabbit hind leg. Under Wagner's assay conditions we observed a  $K_m$  of 17  $\mu M$  and a  $V_{max}$  of 10  $s^{-1}$  for our preparation, which were intermediate to his S-1-A1 and S-1-A2 values. In our assay, the S-1 alone had a small but measurable ATPase activity of its own. This value (0.035  $\mu mole Pi/min/mg$ ) becomes significant in experiments where the S-1 levels are high relative to those of actin. This endogenous activity has been subtracted from all data where it contributes greater than 5% to the initial actin-activated rates.

Finally, control experiments at four different S-1 to actin ratios indicated that cardiac Tm and skeletal  $\alpha$ -Tm (which had been purified using 8 M urea) had the identical abilities to function as regulatory proteins in our ATPase system, both in the presence and in the absence of calcium.

### C. RESULTS.

Keeping these facts in mind, it was possible to obtain some consistent and reproducible data with this assay system. For each of the following graphs the S-1 and actin concentrations were kept constant (details are given in the figure legends). The ATP hydrolysis rate in the absence of regulatory proteins was taken first and constituted the 100 value. Tropomyosin was then added to the assay, and its effect on this hydrolysis rate was recorded. A separate assay was carried out for each of the Tm concentrations shown in the following figures. The Tm effect was recorded in half-shaded symbols in Figs. 25, 26, 27 and 28. After tropomyosin, troponin was added to the assays. The Tn concentration was kept constant at 1 to 7 with respect to actin in all of the experiments. The Tm-Tn inhibition is illustrated in all of the figures with solid symbols. Finally, 0.2 mM calcium was added to the assay (giving a 0.1 mM "free"  $\text{Ca}^{2+}$  concentration) in order to give an estimate of the calcium sensitive release of inhibition. These values have been recorded with the open symbols. One assay then, gives three points at each Tm concentration in the figures.

Our first experiments were done using a molar ratio of S-1 to actin of 1 to 2. Under these conditions Côté (1980) had previously shown that good inhibition and release of inhibition could be obtained. He also had noted that potentiation of the ATPase occurred at the lower ratios of Tm to actin. Figure 25 illustrates the results of an  $\alpha$ -Tm "titration" experiment. The tropomyosin inhibition alone is good, approaching the 45 value (55%) when the molar ratio of Tm to actin exceeds 0.14. After this point the inhibition levels off. The troponin gives an added inhibition which is more noticeable when only a small amount of tropomyosin had been added. The first point on the graph (where Tm added is zero) provides a

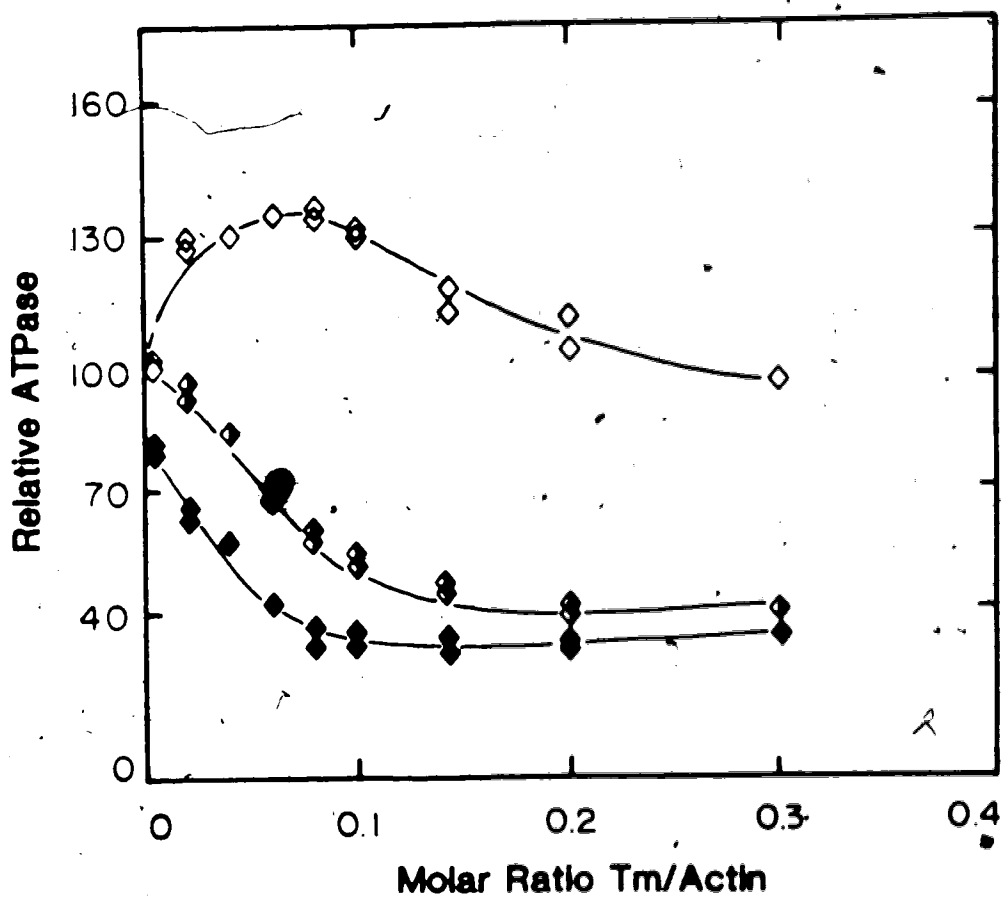


Fig. 25. Effect of  $\alpha$ -tropomyosin on the actin-activated ATPase of S-1. The assay contained  $3 \mu\text{M}$  S-1,  $6 \mu\text{M}$  actin and  $0.857 \mu\text{M}$  troponin.  $\alpha$ -Tm was varied between 0 and  $0.18 \mu\text{M}$ . Half-shaded symbols represent Tm effect alone on the ATPase activity in the absence of calcium, solid symbols represent the Tm-Tn inhibition and open symbols represent the activity in the presence of calcium. Assay buffer contained  $30 \text{ mM}$  KCl,  $0.1 \text{ mM}$  EGTA,  $5 \text{ mM}$   $\text{CaCl}_2$ ,  $2 \text{ mM}$  ATP and  $2 \text{ mM}$  Tris pH 7.8. The ATPase activity of S-1 and actin alone (100 mark) is  $0.311 \mu\text{mol Pi/min/mg S-1}$  ( $0.613 \text{ s}^{-1}$ ).

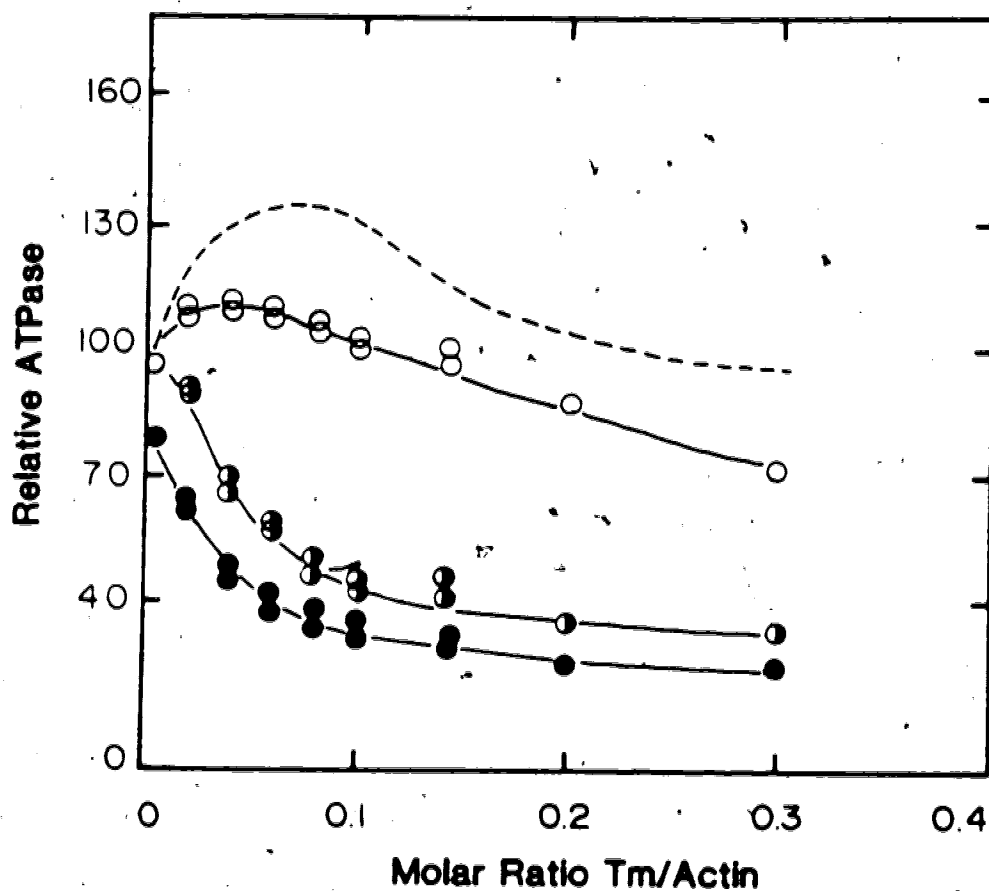


Fig. 26. Effect of  $\beta$ -tropomyosin on the actin-activated ATPase of S-1. The assay contained  $3 \mu\text{M}$  S-1,  $6 \mu\text{M}$  actin and  $0.857 \mu\text{M}$  troponin.  $\beta$ -Tm was varied between 0 and  $0.18 \mu\text{M}$ . Half-shaded symbols represent Tm effect alone on the ATPase activity in the absence of calcium, solid symbols represent the Tm-Tn inhibition and open symbols represent the activity in the presence of calcium. Assay buffer contained  $30 \text{ mM}$  KCl,  $0.1 \text{ mM}$  EGTA,  $5 \text{ mM}$   $\text{CaCl}_2$ ,  $2 \text{ mM}$  ATP and  $2 \text{ mM}$  Tris pH 7.8. The ATPase activity of S-1 and actin alone (100 mark) is  $0.311 \mu\text{mol Pi/min/mg S-1}$  ( $0.613 \text{ s}^{-1}$ ).

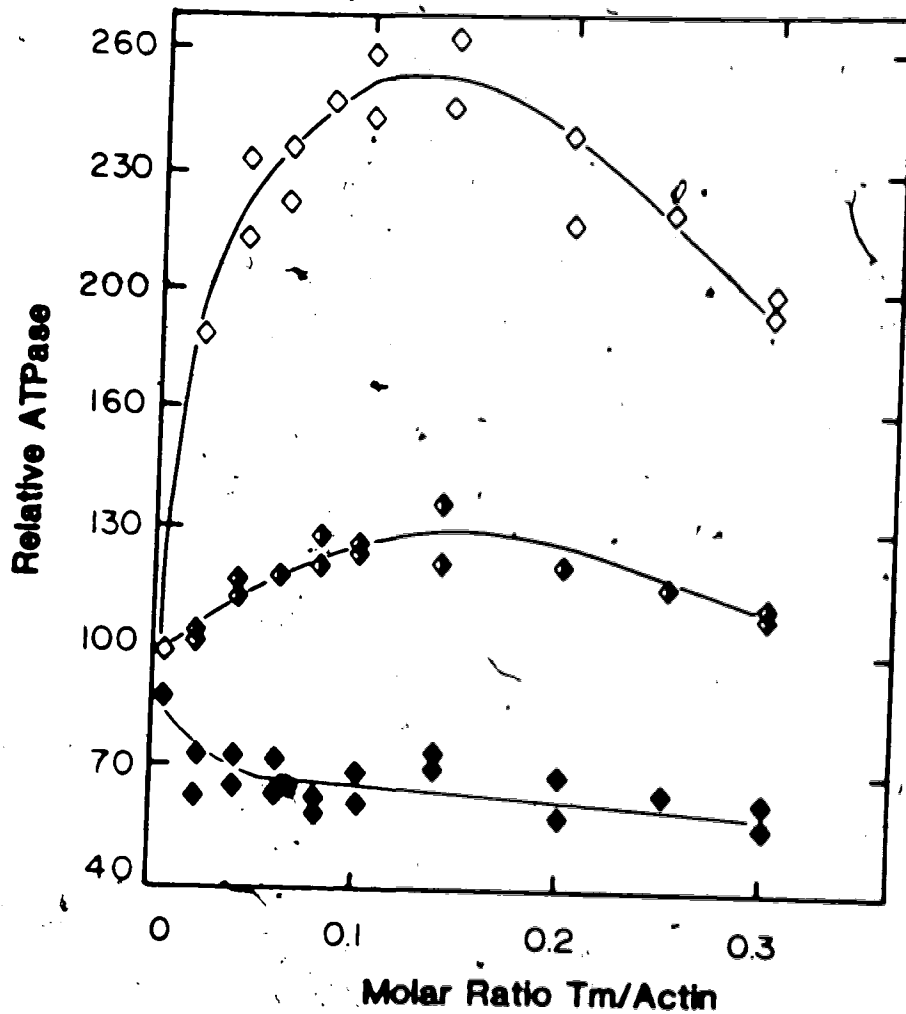


Fig. 27. Effect of  $\alpha$ -tropomyosin on the actin-activated ATPase of S-1. The assay contained  $6 \mu\text{M}$  S-1,  $3 \mu\text{M}$  actin and  $0.428 \mu\text{M}$  troponin.  $\alpha$ -Tm was varied between 0 and  $0.9 \mu\text{M}$ . Half shaded symbols represent Tm effect alone on the ATPase in the absence of calcium, solid symbols represent the Tm-Tn inhibition and open symbols represent the activity in the presence of calcium. Assay buffer contained  $30 \text{ mM}$  KCl,  $0.1 \text{ mM}$  EGTA,  $5 \text{ mM}$   $\text{CaCl}_2$ ,  $2 \text{ mM}$  ATP and  $2 \text{ mM}$  Tris pH 7.8. The ATPase activity of S-1 and actin alone (100 mark) is  $0.277 \mu\text{mol Pi/min/mg S-1}$  ( $0.547 \text{ s}^{-1}$ ).

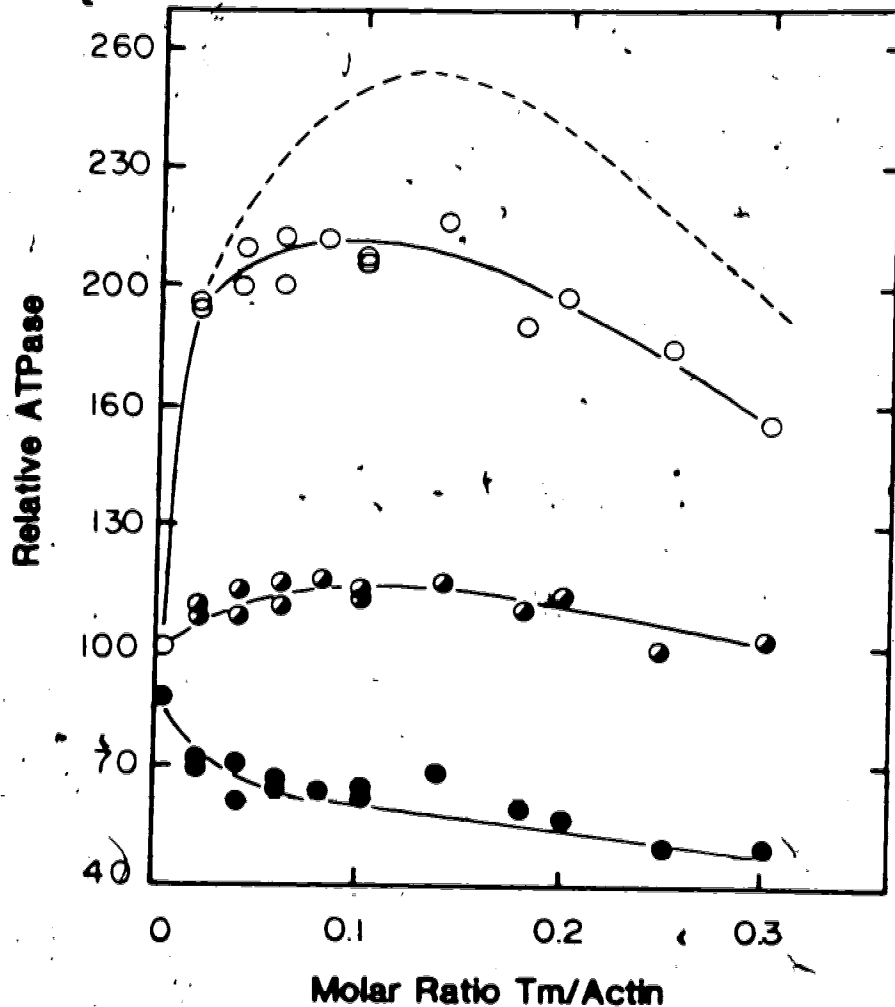


Fig. 28. Effect of  $\beta$ -tropomyosin on the actin-activated ATPase of S-1. The assay contained  $6 \mu\text{M}$  S-1,  $3 \mu\text{M}$  actin and  $0.428 \mu\text{M}$  troponin.  $\beta$ -Tm was varied between 0 and  $0.9 \mu\text{M}$ . Half shaded symbols represent Tm effect alone on the ATPase activity in the absence of calcium, solid symbols represent the Tm-Tn inhibition and open symbols represent the activity in the presence of calcium. Assay buffer contained  $30 \text{ mM}$  KCl,  $0.1 \text{ mM}$  EGTA,  $5 \text{ mM}$   $\text{CaCl}_2$ ,  $2 \text{ mM}$  ATP and  $2 \text{ mM}$  Tris pH 7.8. The ATPase activity of S-1 and actin alone (100 mark) is  $0.277 \mu\text{mol Pi/min/mg S-1}$  ( $0.547 \text{ s}^{-1}$ ).

a control for the experiment. Here it can be seen that troponin alone can give a 15% calcium sensitive inhibition of the ATPase activity (Potter and Gergley, 1974).

In the presence of calcium we saw a potentiation for  $\alpha$ -tropomyosin of 35% (135 value) over that of the original ATPase activity. At the higher levels of tropomyosin to actin, this potentiation falls off slightly. At the present time we do not know what causes this lowering of activity.

Figure 26 illustrates the results for  $\beta$ -Tm under the same experimental conditions. The  $\alpha$ -Tm potentiation values from Fig. 25 have been recorded here (----) for comparison. The inhibition profiles for  $\beta$ -Tm as compared to the  $\alpha$ -form both in the presence and absence of troponin were essentially identical. However, upon addition of calcium, the  $\beta$ -Tm was less able to potentiate the ATPase (only 10% as compared with 35%). As the two experiments had been performed on the same day, and with the same S-1, actin and troponin preparations, we felt that our recorded differences were valid. The question now asked was whether or not the amplification could be increased at yet higher levels of S-1 to actin. Figures 27 and 28 were done with a molar ratio of S-1 to actin of 2 to 1. Fig. 27 illustrates the results with  $\alpha$ -Tm. First of all, the addition of Tm alone to the acto-S-1 resulted not in inhibition, but in a slight (25-35%) potentiation. That Tm alone can potentiate the acto-S-1 ATPase had been observed previously by a number of workers (Bremel et al., 1972, Eaton et al., 1975 and Shigekawa and Tonomura, 1973), so was not a novel observation. The addition of troponin resulted in a net inhibition; however this value (40%) was less than in Fig. 25 (65%) at the lower S-1 to actin ratio. There seemed to be a correlation, then, between increasing S-1 levels and a decrease in the ability of the regulatory proteins



to inhibit the ATPase. The most startling results, though, were the  $\text{Ca}^{2+}$  activated values. For  $\alpha$ -Tm the calcium sensitive release of inhibition approached a value 150% higher than the original acto-S-1 activity. As more Tm was added, these potentiated values became less pronounced (as in Fig. 25).

Figure 28 illustrates the  $\beta$ -Tm curve under the same conditions.  $\beta$ -Tm added alone to the assay gave a slight potentiation of the ATPase activity but this value was slightly less than for  $\alpha$ -Tm (10% as compared with 25 to 35% for  $\alpha$ -Tm). The addition of troponin to the system resulted in an inhibition by Tm-Tm of 40% (in agreement with the previous values for  $\alpha$ -Tm). Finally, the addition of calcium resulted in a potentiation of 110%. This was considerably less than was observed for  $\alpha$ -Tm (as illustrated by the dotted line). Where the two tropomyosins differed by only 20% at the lower ratios of S-1 to actin (Figs. 25 and 26) they now differed by 40%. It seems, then, that the higher the S-1 to actin ratios are, the more "amplified" the differences between the two forms of tropomyosin become. This would predict that at lower S-1 to actin ratios the two forms would become more alike in their abilities to respond to the calcium signal.

Fig 29 illustrates an experiment done with both tropomyosins under conditions of one S-1 per seven actin monomers. As can be seen, the inhibition of both tropomyosin forms is similar, and so is their ability to release this inhibition in the presence of calcium. No potentiation of the ATP hydrolysis occurred for either of the Tms at any point in the curves in the presence of calcium.

Once our assay system had been set up, it was of interest for us

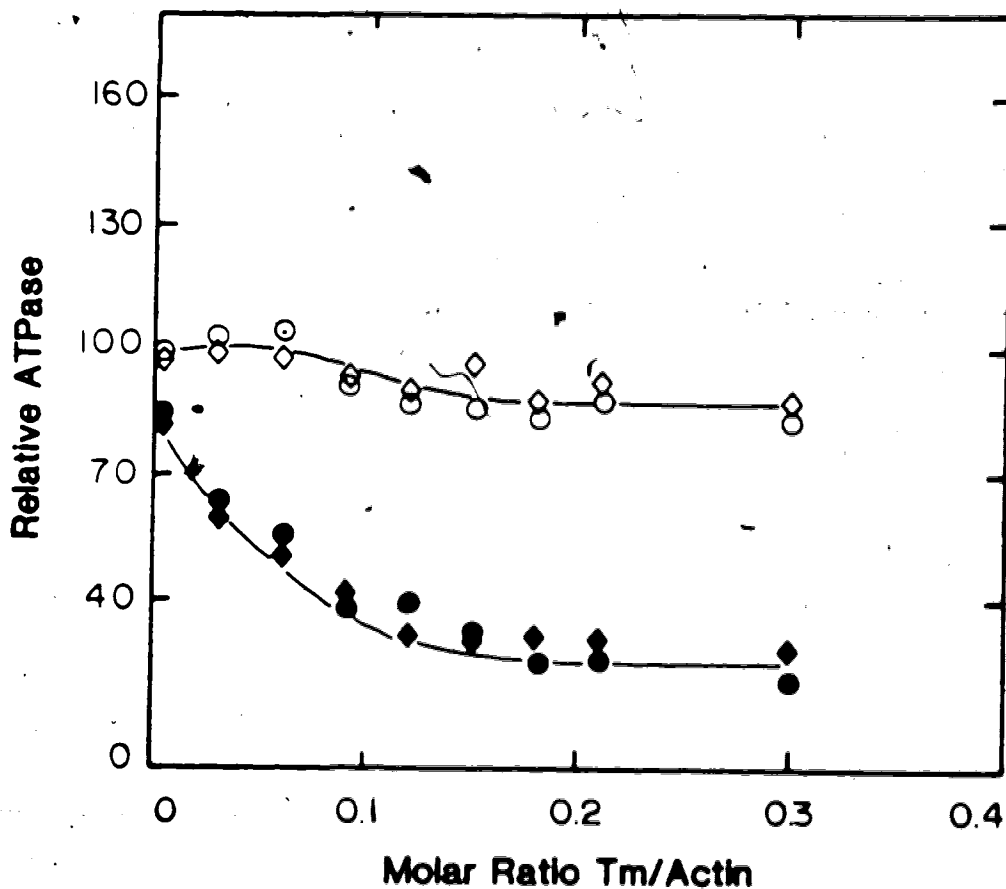


Fig. 29. Effect of  $\alpha$  ( $\diamond$ ) and  $\beta$  ( $\circ$ ) tropomyosins on the actin-activated ATPase of S-1. The assay contained 1.28  $\mu$ M S-1, 9  $\mu$ M actin and 1.28  $\mu$ M troponin. Tropomyosins were varied between 0 and 0.27  $\mu$ M. Solid symbols represent the Tm-Tn inhibition and open symbols represent the activity in the presence of calcium. Assay buffer contained 30 mM KCl, 0.1 mM EGTA, 5 mM  $\text{CaCl}_2$ , 2 mM ATP and 2 mM Tris pH 7.8. The ATPase activity of S-1 and actin alone (100 mark) is 0.673  $\mu$ mol Pi/min/mg S-1 ( $1.33 \text{ s}^{-1}$ ).

to see how non-polymerizable tropomyosin (NPTm) would function as a replacement for normal tropomyosin. We chose to do the experiments at an S-1 to actin ratio of 1 to 1, hoping for reasonable levels of inhibition and potentiation. Fig. 30 is the  $\alpha$ -Tm control curve. Inhibition in the presence of troponin was good, (65-70%) and calcium sensitive release of inhibition gave a 60% potentiation of the ATPase, which was intermediate to the values obtained with the 2 to 1 (160%) and 1 to 2 (30%) molar ratios of S-1 to actin. NPTm under these conditions behaved entirely differently. First of all, when NPTm was added to the S-1 and actin in the presence of ATP, it had no effect at all on the hydrolysis rate. This was not entirely unexpected since NPTm binds negligibly to F-actin under conditions where  $\alpha$ -Tm binding is maximal (Mak and Smillie, 1981a), and as such should not be expected to affect acto-S-1 interactions. The addition of troponin brought the inhibition down to within 10% of the  $\alpha$ -Tm values (see Fig. 31). The subsequent addition of calcium released the NPTm-Tn inhibition completely; however, no activation was seen. The rate of the ATP hydrolysis was essentially the same as for unregulated actin filaments.

Since the process of preparing NPTm (Mak and Smillie, 1981a) involved heating the digested tropomyosin for 3 minutes at 85°C (in order to inactivate the carboxypeptidase A) it was important for us to see how heat treatment might affect Tm's biological activity. Cummins and Perry (1973) had shown that heat denaturation of tropomyosin (60°C for 70 min. or 100°C for 10 min.) would affect the ability of Tm to inhibit the  $Mg^{2+}$ -stimulated desensitized actomyosin ATPase (see discussion for details) by over 80%. We decided to test the conditions used in making NPTm on samples of  $\alpha$ -tropomyosin in our actin-S-1 system. We chose an S-1 to

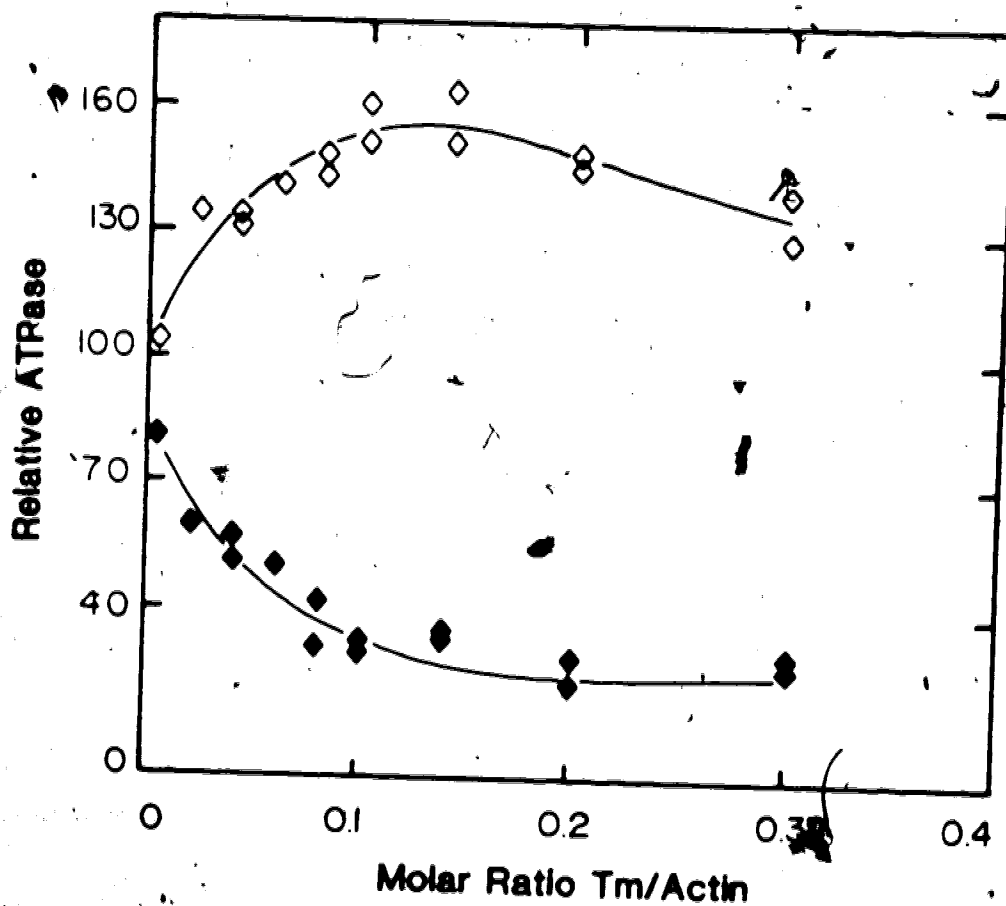


Fig. 30. Effect of  $\alpha$ -tropomyosin on the actin-activated ATPase of S-1. The assay contained  $3 \mu\text{M}$  S-1,  $3 \mu\text{M}$  actin and  $0.428 \mu\text{M}$  troponin.  $\alpha$ -Tm was varied between 0 and  $0.9 \mu\text{M}$ . Solid symbols represent the Tm-Tn inhibition and open symbols represent the activity in the presence of calcium. Assay buffer contained  $30 \text{ mM}$  KCl,  $0.1 \text{ mM}$  EGTA,  $5 \text{ mM}$  CaCl<sub>2</sub>,  $2 \text{ mM}$  ATP and  $2 \text{ mM}$  Tris pH 7.8. The ATPase activity of S-1 and actin alone (100 mark) is  $0.277 \mu\text{mol Pi/min/mg S-1}$  ( $0.546 \text{ s}^{-1}$ ).

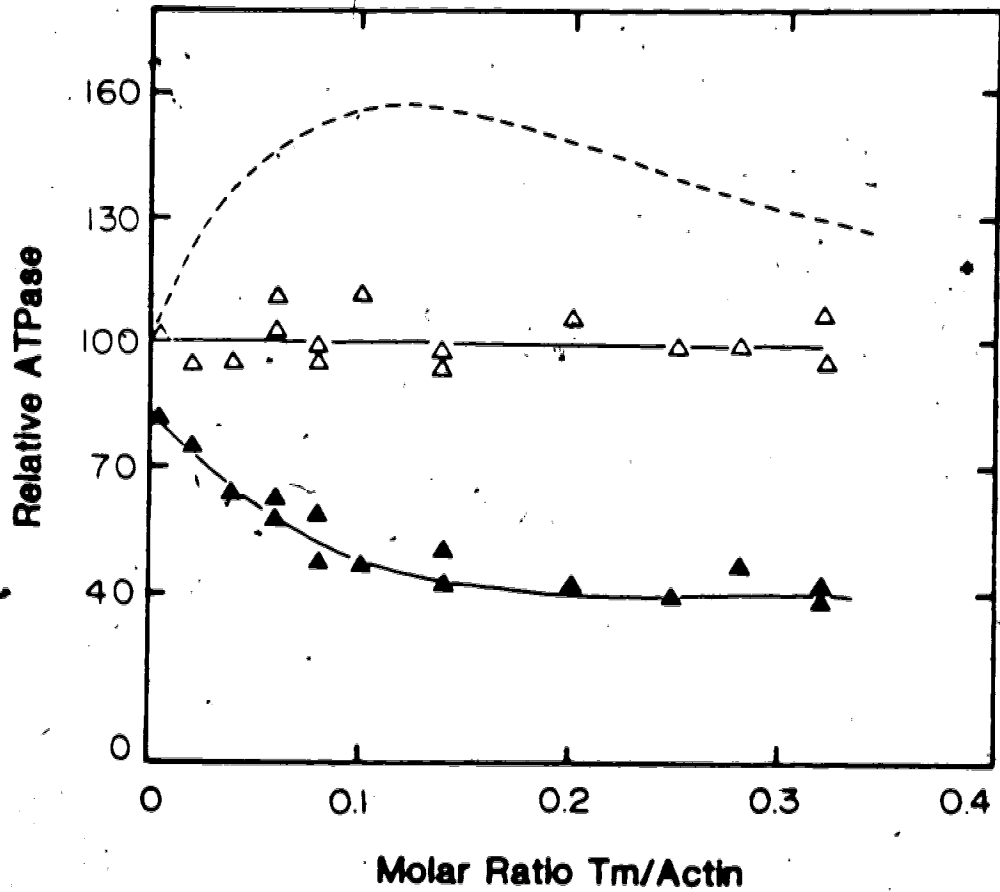


Fig. 31. Effect of NPTm on the actin-activated ATPase of S-1. The assay contained  $3 \mu\text{M}$  S-1,  $3 \mu\text{M}$  actin and  $0.428 \mu\text{M}$  troponin. NPTm was varied between 0 and  $0.9 \mu\text{M}$ . Solid symbols represent the NPTm-Tn inhibition and open symbols represent the activity in the presence of calcium. Assay buffer contained  $30 \text{ mM}$  KCl,  $0.1 \text{ mM}$  EGTA,  $5 \text{ mM}$   $\text{CaCl}_2$ ,  $2 \text{ mM}$  ATP and  $2 \text{ mM}$  Tris pH 7.8. The ATPase activity of S-1 and actin alone (100 mark) is  $0.277 \mu\text{mol Pi}/\text{min}/\text{mg S-1}$  ( $0.546 \text{ s}^{-1}$ ).

actin ratio of 1 to 1. The  $\alpha$ -tropomyosin sample (1.25 mg/ml in ATPase buffer plus 2 mM DTT) was heated in a water bath at 85°C and at timed intervals samples were withdrawn. These samples were allowed to come to room temperature before being assayed in the usual manner. After 20 minutes at 85°C, the remaining tropomyosin was boiled for an additional 10 minutes. It was assayed after cooling. The results in the table below show that heat treatment of tropomyosin in our assay system had no effect

the ability of Tm to function as a regulatory protein, either in inhibiting or in activating the acto-S-1 ATPase activity. The results show that the thermal denaturation of tropomyosin is reversible.

<u>Temperature °C</u>	<u>Time (min.)</u>	<u>%Inhibition</u>	<u>%Potentiation</u>
85	3	51	56
85	8	58	46
85	15	54	45
85	20	58	40
100	10	49	43

The results in this chapter have been verified with two different S-1 preparations, and numerous actin and Tm subunit preparations, so the differences we see between  $\alpha$  and  $\beta$ -tropomyosins are not artifacts due to one particular protein batch. Troponin was the one exception. For all the ATPase experiments reported here, only one preparation of Tn was used. Troponin is a complex of three polypeptide chains and their ratios tend to vary from preparation to preparation, regardless of the care taken in keeping the purification procedure consistent. Batches higher in the Tn-T subunit were found to be less capable of giving 100% release of inhibition at S-1 to actin ratios of 1 to 2 with Tm and Tn present as 1 to 7 ratios with respect to the actin concentration (standard conditions). The  $\text{Ca}^{2+}$  sensitivities of several troponins were

tested and the one that gave the best release of inhibition was used for all the assays. We considered this functional test to be the most reliable one for our choice of a suitable troponin preparation.

#### D. DISCUSSION

In this chapter,  $\alpha$  and  $\beta$ -tropomyosins were compared with respect to their abilities to function in a reconstituted muscle system. The assay conditions of 3 mM free  $Mg^{2+}$  and 30 mM KCl were chosen since the reconstituted acto-S-1 Tm-Tn system exhibited good calcium sensitivity under these conditions, and permitted the ATPase assays to be carried out in a reproducible manner.

The biological activity of  $\alpha$  and  $\beta$ -tropomyosins had been previously tested by Cummins and Perry (1973) using desensitized actomyosin. In an assay system of this type it is difficult to obtain a quantitative estimate of the ratio of actin to myosin since the proteins have not been separately purified and reconstituted in known amounts. As well, it is difficult to be sure that all of the pre-existing regulatory proteins have been removed. The conditions these workers used for the assay (25 mM Tris-HCl pH 7.6, 2.5 mM ATP, 2.5 mM  $MgCl_2$  and 1 mM EGTA) also cast doubt as to how well the Tm would be able to bind to actin. Our binding studies in chapter 3 had shown us that at least 1 to 1.5 mM "free"  $Mg^{2+}$  must exist for maximal binding to occur. Under the experimental conditions above, all of the magnesium ions would be bound up as Mg-ATP. Finally, although Cummins and Perry had studied the effect of  $\alpha$  and  $\beta$ -Tms as inhibitors of the ATPase activity (both in the presence and absence of troponin), no study had ever been undertaken to see how well the tropomyosins functioned in the presence of troponin and calcium, where release of inhibition occurs. For these reasons we undertook a reinvesti-

gation of this area using an acto-S-1 ATPase system which had been carefully tested in control experiments.

Skeletal tropomyosin has been postulated to bind in two positions on the F-actin filament; near the periphery (inhibition) and closer to the center of the groove (activation) (Haselgrove, 1972; Huxley, 1972 and Wakabayashi et al., 1975). In this model system Tm would function to sterically block the myosin head (as originally suggested by the data of Moore et al., 1970; challenged by Seymour and O'Brien, 1980; reaffirmed by Taylor and Amos, 1981. See Squire, 1981 for an overview). Alternately, a movement of Tm could cause a conformational change in the F-actin monomers, making them more or less receptive to the myosin heads.

Our studies indicate that at low ratios of S-1 to actin, tropomyosin binds preferentially in the inhibitory position. For instance, at an S-1 to actin ratio of 1 to 7, both  $\alpha$  and  $\beta$ -tropomyosins alone inhibit the ATPase activity by 60%. The addition of troponin gives rise to an extra added inhibition, especially at the lower ratios of tropomyosin (less than 0.142 to 1 with respect to actin) where the filaments are not yet "saturated" with Tm. Troponin, possibly through its interaction with actin via Tn-I, provides an additional stabilization for Tm in the inhibitory state, especially noticeable in Figs. 27 and 28.

At the higher levels of S-1 to actin (greater than 1 to 1) Tm alone is no longer capable of binding in the inhibitory position, possibly because of the fact that at any one time a high concentration of myosin intermediates are interacting favourably with the F-actin monomers. In this case, Tm would be induced to bind closer in the groove in the activating position. Once it does, however, its binding stabilizes the filaments and the activity is potentiated (see Figs. 27 and 28).



Thus we have a synergistic response; myosin forces tropomyosin into the activating position and this binding in turn facilitates myosin-actin interactions. The addition of troponin is partially able to counteract the myosin effect and results in an inhibitory response, but this inhibition is now weaker (40%) than at the lower ratios of S-1 to actin (65-70%).

Overall then, the interaction between Tm, actin and myosin heads seems to depend in a complex manner on their relative concentrations (inhibition decreases as S-1 to actin ratios increase) and also on the nature of the proteins themselves. For instance, Yang et al., (1977) have shown that Acanthamoeba actin with skeletal myosin shows activation upon the addition of skeletal Tm under conditions where skeletal Tm inhibits skeletal actomyosin. As well, Sobieszek and Small (1977) have shown that smooth muscle Tm alone is capable of activating the actomyosin ATPase under conditions where skeletal Tm does not.

In the presence of calcium and troponin our results show that a release of inhibition occurs in all cases. At low ratios of S-1 to actin (1 to 7) this release gives back approximately 100% of the original unregulated value, but at higher levels of S-1 to actin, potentiation of the ATPase activity is seen. At these potentiated levels the differences between  $\alpha$  and  $\beta$ -tropomyosins are obvious.

Potentiation by rigor complexes at low ATP levels and by high concentrations of S-1 at high levels of ATP has been well documented (Bremel et al., 1972; Shigekawa and Tonomura, 1973). Based on these observations Murray and Weber (1980a,b) proposed that a competition exists between S-1 and tropomyosin for actin binding sites. This competition is in Tm's favour when the S-1 to actin ratios are low. Because Tn enhan-

ces tropomyosin's binding to the peripheral sites, it increases the ability for Tm to compete effectively with S-1. According to this model,  $\beta$ -Tm would appear to be a better "competitor" for myosin binding sites than  $\alpha$ -Tm. Murray and Weber propose that potentiation occurs when the tropomyosin filaments are completely shifted into the grooves when a sufficiently high degree of myosin saturation occurs. This shift (with the cooperation of the bound myosin and Tm) causes a conformational change in the 7 actin monomers thereby increasing their affinity for myosin (lower  $K_m$ ). Because the Murray and Weber model is based on the steric blocking model; it predicts that in the absence of calcium tropomyosin should block access to the S-1-ATP intermediates (poor competitors) and weaken the affinity of rigor complexes (good competitors) 10-fold (Murray *et al.*, 1980a). These predictions, however, are in direct contrast to the recent results from the binding studies of Greene and Eisenberg (1980) and the stopped flow absorbance studies of Chalovich *et al.* (1981). These workers showed that the S-1-ATP or S-1-ADP-Pi intermediates were able to bind with approximately the same binding constant to both regulated and unregulated actin filaments. The binding of S-1-ADP on the other hand, was affected by Tm-Tn. Initially the rigor complex bound very weakly to regulated filaments, but as its occupancy increased, a cooperative change was manifested and the actin filaments switched from a weak to a strong binding form. At high levels of occupancy S-1-ADP bound three times as strongly as to unregulated actin filaments. This same phenomenon was shown to occur in the presence of calcium (where the steric blocking predicts complete access); however the transition from the weak to strong binding forms occurred sooner. The regulated actin filament, then, was seen to occur in two states, with calcium and S-1-ADP acting as allosteric effect-

ors between them. Instead of blocking myosin access to these filaments, tropomyosin was postulated to alter the rate of product release (the 90° to 45° transition). Whether Tm does this physically by itself or through a conformational change in the F-actin monomers is not known at the present time.

With relation to the ATPase, then, it would seem that the strong binding form would correspond to the potentiated active state and the weak binding form would correspond to the inhibited state. Since  $\alpha$  and  $\beta$ -Tms show differences mainly in their abilities to potentiate the ATPase activity of the acto-S-1 system, it would appear that they differ in their abilities to allow the transition between weak to strong binding forms of actin to occur. Is this because  $\beta$ -Tm has a stronger ability to associate with F-actin, or a weaker ability to associate with troponin (which in turn might affect its head to tail interactions)? Which of these variables (or which combination of them) gives rise to the differences which we observe in our ATPase studies?

The results in chapter 3 have shown us that the F-actin affinity for  $\beta$ -Tm is stronger than that of  $\alpha$ -Tm at the higher ionic strengths. Are these findings relevant to our assay conditions? Mak et al., (1980) have compared the sequences of  $\alpha$  and  $\beta$ -tropomyosins. Since most of the amino acid substitutions are conservative, the periodicities corresponding to actin binding sites (McLachlan and Stewart, 1976a) are also found in  $\beta$ -Tm, essentially without a change. The prediction put forward at the time was that no major differences between the two forms of Tm would occur. Work by Wegner (1979) showed that the binding of tropomyosin to F-actin filaments (as determined by light scattering methods) is highly cooperative. Single Tm molecules have a very weak affinity for the actin but

through head to tail overlap of neighbouring molecules, this affinity is greatly enhanced. These observations were extended (Wegner, 1980) to  $\alpha,\alpha$ -homodimers ( $\alpha$ -Tm) and  $\alpha,\beta$ -heterodimers of tropomyosin. Under the binding conditions of 80 mM KCl, 1 mM EGTA and temperatures between 38 and 41°C, very little difference in the relative affinities of the two forms of Tm for the actin filaments was seen.  $K$  (the affinity constant for single tropomyosin molecules for the actin filaments) is  $4.6 \times 10^3 \text{ M}^{-1}$  for the  $\alpha,\alpha$ -homodimers and  $4.7 \times 10^3 \text{ M}^{-1}$  for the  $\alpha,\beta$ -heterodimers at 39.3°C. This constant  $K$  does not take into account the head to tail overlap contribution of neighbouring molecules. Thus it is a good indication of how the Tm molecules interact with actin through their  $\alpha$  and  $\beta$ -bands. The differences between  $\alpha,\alpha$  and  $\alpha,\beta$ -Tm dimers in this respect is small (at 80 mM KCl). Wegner (1980) also obtained a value  $\omega$  which represents the affinity of the tropomyosin for head to tail overlap. This value is also very similar (250 for  $\alpha,\alpha$ -Tm and 200 for  $\alpha,\beta$ -Tm) at 39.4°C. Unfortunately, similar measurements with  $\beta,\beta$ -homodimers ( $\beta$ -Tm) were not reported.

If Wegner's binding studies could be repeated at the higher ionic strengths we would be able to see if the differences in our co-sedimentation experiments are due to differences in the  $\alpha$  and  $\beta$ -tropomyosin actin binding sites or if they are mainly a reflection of an altered ability for the tropomyosin molecules to make end to end contact. Although our viscosity studies show that most of the head to tail aggregation (for both forms of Tm) falls off at 0.2 M KCl, it does not prove that all head to tail overlap is abolished. Since polymerization can strengthen tropomyosin's binding affinity for F-actin 1000 fold, it is hard to know if this variable contributes to the actin binding ability of Tm molecules at the higher ionic strengths.

Our ATPase conditions had been selected to give both forms of tropomyosin an equal chance of interacting with F-actin. Although the evidence is incomplete (e.g.; the effects of S-1 on the interactions between  $\alpha$  and  $\beta$ -tropomyosin and the actin filaments had not been investigated), the present indications are that there is little difference in the binding of the two forms at the lower ionic strengths in the presence of 3 mM free  $Mg^{2+}$ .

It is obvious from the non-polymerizable tropomyosin binding studies of Mak and Smillie (1981a) that head to tail overlap is essential for strong actin binding. Removal of just 11 amino acid residues from the COOH-terminal end of the tropomyosin molecule almost entirely abolishes its capacity to interact with actin. These results agree with Wegner's (1980) observations that single Tm molecules have a weak affinity for F-actin. Thus the ability of  $\alpha$  and  $\beta$ -Tms to regulate the acto-S-1 ATPase activity may be more dependent on the nature of their head to tail overlap than their actin binding abilities.

Our viscosity studies, however, have shown little difference between  $\alpha$  and  $\beta$ -tropomyosins in their ability to polymerize. Since viscosity has also not indicated any differences between the two tropomyosin forms in their ability to bind troponin (whereas affinity chromatography and gel filtration studies did), this technique may not be sensitive enough for our purposes. Our ATPase studies at a 2 to 1 molar ratio of S-1 to actin indicate that  $\alpha$ -Tm (Fig. 27; half shaded symbols) alone can potentiate the ATPase activity by 25-35% whereas  $\beta$ -Tm (Fig. 28) is less able to do so (10%). Since troponin is not present in these measurements, the results would suggest that differences in the Tm head to tail overlap region may be responsible for the results.

Further studies at still higher ratios of S-1 to actin should be attempted in the future to amplify these differences, if possible. As well, other studies could be conducted in which the COOH-terminal fragments of the two forms of Tm would be passed through  $\alpha$  or  $\beta$ -tropomyosin affinity columns. Elution of these fragments may give us a better indication of the differences in their head to tail polymerization abilities.

Finally we must consider how the troponin-binding ability of the two forms of Tm may affect their abilities to function in the reconstituted ATPase system. Since troponin binding to Tm can affect the head to tail region (Fig. 16) (either by direct interaction or by the perturbation of a conformational change along the Tm filaments), it is likely that this binding will have some consequences in the ability of the Tm units to function cooperatively in the ATPase assay. The studies of Mak and Smillie (1981b) give evidence for a Tn-T calcium insensitive binding site on the tropomyosin molecule near its COOH-terminal region (residues 258-284). Our gel filtration studies with the Tn-T fragment CBI (which is the portion of the Tn-T subunit that interacts with Tm in a calcium insensitive manner) have shown definite differences between the  $\alpha$  and  $\beta$ -tropomyosins in their abilities to interact with this fragment. CBI binding as a ternary complex with COOH and NH<sub>2</sub>-terminal fragments of  $\alpha$ -Tm has been demonstrated to enhance their head to tail interactions (Pato *et al.*, 1981). This would suggest (but does not prove) that a weaker association of CBI (and whole troponin) to  $\beta$ -Tm would affect its ability to function cooperatively.

ATPase studies in this chapter with NPTm confirm the fact that potentiation is dependent on the cooperative interactions involving the head to tail overlap regions of the Tm molecules. The removal of the ter-

minimal 11 amino acid residues completely abolishes the inhibitory capacity of this molecule (the ATPase rate is not altered by the addition of NPTm into the assay mixture). This would imply that the bulk of the tropomyosin molecule has a very weak association with the actin filaments under our ATPase conditions (even in the presence of 3mM "free"  $Mg^{2+}$ ), and that strong binding requires head to tail overlap or the presence of troponin. Indeed, the addition of Tn to the NPTm, actin and S-1 results in a significant amount of inhibition. NPTm does not associate well with CBI under physiological conditions (Mak and Smillie, 1981b). Nevertheless, there is a second, calcium sensitive Tm binding site on Tn-T (residues 197 to 259) which is postulated to bind near the Cys 190 region of the tropomyosin molecule. Since this portion of NPTm has not been affected by the carboxypeptidase digestion, some affinity for the Tn-T portion of troponin will remain. The Tn-I subunit of Tn interacts with actin and stabilizes the non-polymerizable tropomyosin of the F-actin filaments. When  $Ca^{2+}$  is added to the now inhibited assay, release of inhibition occurs (but no potentiation). Since the Tn-I binding site is  $Ca^{2+}$  sensitive (in whole Tn), it is not likely that the NPTm-Tn complex will stay associated with these filaments (or that Tn and NPTm will stay complexed). The S-1 heads are allowed full access to the essentially unregulated actin monomers, and 100% (and only 100%) of the activity is regained.

In summary, then, it seems that head to tail overlap (and how this may be affected through troponin binding) is essential for potentiation of the ATPase. The fact that  $\beta$ -Tm is less able to potentiate the ATPase relative to  $\alpha$ -Tm would argue that its ability to associate in a head to tail fashion is altered relative to the  $\alpha$ -form. This is most likely due to  $\beta$ -Tm's weaker association with troponin but may also include an alter-

ed ability to polymerize (although this has not been demonstrated conclusively).

According to Eisenberg and Greene's allosteric model of regulation,  $\beta$ -Tm can be visualized as being less able cooperatively to assist the actin filaments in undergoing the transition from weak binding to strong binding form. Coupled with the ability for  $\beta$ -Tm to associate more strongly with F-actin at the higher ionic strengths, our investigations on the whole have shown that  $\beta$ -Tm is slightly less of a "regulatory" protein and slightly more of a structural one. Since embryonic muscles and adult slow muscles do not need the quick responses of the adult fast muscles, their higher  $\beta$ -content may be more efficient in the long run (less energy wasted). The  $\beta$ -Tm content of a muscle may be one way to "finetune" it to the organism's requirements. As well, during early development the  $\beta$ -Tm may be more effective in organizing and stabilizing the newly developing thin filaments.

Although highly speculative, these are some explanations for the existence of two tropomyosin gene products in rabbit skeletal muscle.



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