Chapter One: Introduction.

One of the main features distinguishing plants from animals is the ability of animals to move from one place to another. This ability results from the existence of a specialised tissue - muscle. Movement is crucial to many animals' survival: it enables them to seek out food, escape from predators and move to new habitats. However, muscle is not only important in whole body locomotion. Many functions of an animal require the carefully controlled contraction of particular muscles - the chewing of food, the movements of the eye and the ejaculation of sperm being just a few examples.

For a biochemist, the challenge of muscle is to understand how it functions - and how it is controlled - at a molecular level. The major proteins involved in this process have long been known. More recently, the three-dimensional structures of some have been solved. Along with numerous kinetic studies this has thrown light on how protein machines convert chemical energy to mechanical work, but the details of this process remain obscure. Furthermore, although the proteins involved in regulating the process are known and well characterised the precise manner in which they work together to switch contraction on - and off again - is not known. Nor is it known how different isoforms of these proteins exert subtle effects on the phenotype of muscle tissue permitting such a wide variation in the properties of this versatile tissue.

1.1 THE MOLECULAR BASIS OF MUSCLE CONTRACTION.

1.1.1 Introduction.

When muscle contracts, mechanical work is done. The process is powered by coupling the chemical energy released by the hydrolysis of MgATP (to MgADP and P_i) to structural rearrangements of muscle proteins. The energy transducing enzyme is myosin (see review by Sellars and Goodson, 1995 and references contained therein) which works together with actin to bring about contraction.

1.1.2 Ultrastructural Organisation of Muscle Tissue.

Muscle cells are highly specialised and show many adaptations to their task. Vertebrate striated muscle cells are multi-nucleate, elongated and show many invaginations of the plasma membrane. Closer examination of the cytoplasm (sarcoplasm) by phase contrast or electron microscopy shows many repeating sarcomeres (figure 1.1a). Within these sarcomeres, individual filaments can be distinguished which can be sub-divided into two categories - thick and thin (figure 1.1b). The endoplasmic (sarcoplasmic) reticulum of muscle cells is extensive, whereas most other organelles are fewer in number and smaller than in other cells see (see Squire, 1981).

1.1.3 The Sliding Filament Hypothesis.

The thick and thin filaments of the sarcomere overlap. Some of the earliest observations on living muscle tissue showed that this degree of overlap increases on contraction whereas the length of both sets of filaments remains constant (figure 1.2) (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). The principal protein of the thin filament is actin; the thick filament being composed mainly of myosin. The sliding filament hypothesis holds that myosin filaments pull past actin filaments hydrolysing MgATP in the process, resulting in shortening of the sarcomere and - provided the process is repeated many times - shortening of the muscle cell. The sliding filament hypothesis is now widely accepted with only a small number of detractors eg (Oplatka, 1994).

1.1.4 Structure of the Myosin Molecule.

1.1.4.1 Gross Structural Features.

Myosin is a hexamer of approximately 480 kDa molecular mass, consisting of two heavy chains (each around 200 kDa) and four light chains (of 18-22 kDa). Examination of myosin by electron microscopy, shows it to be an elongated molecule with two globular heads at the N-terminus (figure 1.3a) (Lowey *et al.* 1969). Proteolytic degradation (in high ionic strength buffer and in the presence of magnesium ions)

results in two fragments: heavy (HMM) and light meromyosin (LMM). LMM is insoluble, does not hydrolyse MgATP or bind actin; it does, however retain the ability to form filaments. HMM, on the other hand, is soluble under these conditions, does bind actin and hydrolyse MgATP. When observed by electron microscopy, it is seen to be a double headed structure like myosin, but with a shorter "tail" (Lowey *et al.* 1969).

Digestion of myosin in low ionic strength buffer and with magnesium ions removed using a chelating agent such as EDTA yields different products: insoluble rod and soluble subfragment 1 (S1). It is S1 that retains the actin binding and MgATP hydrolysing activities (Lowey *et al.* 1969). S1 is a globular fragment corresponding to the "heads" seen in the images of myosin. Digestion of HMM under these conditions gives S1 and subfragment 2 (S2). These results are summarised in (figure 1.3b).

Myosin, HMM and S1 all hydrolyse MgATP. The rate is very slow but stimulated by the presence of actin: they are actin-activated MgATPases. This makes sense: it would be a waste of MgATP if myosin were constituitively active. Resources are only consumed when work can be done.

Examination of the primary sequence of the "tail" leads to two interesting observations. For much of its length it follows the pattern that every fourth and seventh amino acid residues are hydrophobic. If the sequence adopts an α-helical structure, then there will be a hydrophobic "stripe" curling gently around the helix. This can be shielded from the aqueous phase if a second α-helix of similar sequence forms a coiled-coil arrangement in which the hydrophobic residues are always on the inside. This is the arrangement in myosin for much of the "rod" (see Sellars and Goodson, 1995; Squire, 1981). The pattern breaks down in three places: at the extreme C-terminus, where the two chains are more disordered (Kalbitzer *et al.* 1991), at the site of cleavage between LMM and HMM where there is a flexible, hinge-like region and where the two chains diverge to form globular heads (this is the site of cleavage between S1 and S2).

Overall, this gives a picture of the myosin molecule in which the N-terminal residues form a compact, globular head (S1) which hydrolyses MgATP and binds actin and the

remainder of the molecule which forms an α-helical coiled coil with a second myosin heavy chain, broken by a single hinge and with a disordered C-terminus. This rod-like section is responsible for the dimerisation of the two heavy chains (S1 is monomeric) and the higher order organisation of filament formation.

1.1.4.2 The Light Chains.

There are four light chains associated with each myosin molecule (see reviews by Matsuda, 1983; Grand, 1982; Trybus, 1994a; Barton, 1985). They fall into two categories: essential light chains (ELC - also termed alkali light chains) and regulatory light chains (RLC - also known as phosphorylatable, DNTB or EDTA light chains). If S1 is produced using papain as a protease, one ELC and one RLC are associated with each S1. Chymotrypsin (which cuts closer to the N-terminus of myosin and results in a shorter fragment) gives S1 with only the ELC associated with it.

Comparison of the primary sequence of the light chains with known sequences shows them to be similar to the calcium binding proteins calmodulin and troponin C, suggesting common ancestry. Like TnC and calmodulin (Klee *et al.* 1980; da Silva and Reinach, 1991), light chains have four putative divalent cation binding sites. However, the ability to bind metal ions has been lost in almost all cases (da Silva and Reinach, 1991). Two exceptions to this are the first binding site of RLCs where a magnesium ion is required for binding of the light chain to the heavy chain and the third site in scallop ELC which binds calcium ions (see section 1.2.4) (Trybus, 1994a). These observations enabled molecular models of the light chains from skeletal muscle to be calculated (Béchet and Houadjeto, 1989). These models showed molecules which could be divided into four sub-domains based around the helix-loop-helix motif of the (non-functional) metal ion binding sites. However, the utility of these models was limited by the lack of knowledge about the light chain-heavy chain binding sites and the solution of the crystal structure of myosin S1 (Rayment *et al.* 1993b) less than eighteen months later.

1.1.4.3 The Crystal Structure of Myosin S1.

S1 was notoriously difficult to crystallise. This is not surprising given that myosin must be a flexible molecule moving between a variety of different conformational states. Rayment and colleagues worked on the problem for over ten years before finally producing crystals following reductive methylation of the majority of the lysine residues (Rayment and Winkelman, 1984). Although this process is reported to result only in minor structural changes in hen egg white lysozyme (Rypniewski *et al.* 1993) it seems likely that - at the very least - it stabilises one of the many structural states of S1 and it is by no means clear which one. The model (Rayment *et al.* 1993b) is subject to further problems: many of the loop regions on the surface of the molecule are mobile and thus undefined. It is these regions that may be involved in interaction with actin and in structural rearrangements through the MgATPase cycle. In addition, the protein used was not pure, but was a mixture of the isoenzymes S1(A1) and S1(A2) (see section 1.4), leading to further uncertainty in the position of residues in the ELC.

Nevertheless, the possession of a plausible model for S1 (figure 1.4) was an enormous step forward in understanding myosin function. Examination of the model shows that it can be considered in two parts: an N-terminal "motor" domain and a C-terminal light chain binding domain. The motor domain contains the MgATP binding site (identified by its similarity to other nucleotide binding sites) and the actin binding sites. Indeed with knowledge of the crystal structure, various workers have been able to express the motor domain and show that it, alone, can generate force (Itakura *et al.* 1993; Waller *et al.* 1995). The coupling of MgATP hydrolysis to mechanical work must begin in this "domain". The process may well be similar to that seen in Ras (Wittinghofer and Pai, 1991) and elongation factor Tu (Berchtold *et al.* 1993; Sprinzl, 1994; Kjeldgaard *et al.* 1993; Kjeldgaard and Nyborg, 1992) where small structural changes resulting from the loss of the γ-phosphate of MgGTP are amplified through the molecule. One of the functions of the light chain binding domain may be to amplify these changes and act as a lever arm. Its structure is quite unusual: it consists of an 8 nm long α-helix, around which are wrapped the two light chains - in a manner that is (superficially) similar to the binding of calmodulin to its target peptide from myosin light chain kinase (Roth *et al.* 1991; Ikura *et al.* 1992) bearing out the predictions (Béchet and Houadjeto, 1989). It is

the longest, uninterrupted stretch of α-helix known in any globular protein - although there is one almost as long in the F_1F_o-ATP synthase of bovine mitochondria (Abrahams *et al.* 1993; Abrahams *et al.* 1994) suggesting that such structures may have a common rôle in energy transducing enzymes. The principal function of the light chains is undoubtedly to stabilise this α-helix that would, otherwise, be exposed to solvent.

The RLC lies at the C-terminus of S1 with the ELC binding adjacent to it. Of these two the ELC-heavy chain complex most closely resembles the structure of Ca^{2+}-calmodulin-MLCK(M13 peptide) with the RLC being more elongated along the heavy chain. The sites on the heavy chain that interact with the light chains conform to a consensus sequence known as the IQ motif (Mercer *et al.* 1991; Cheney and Mooseker, 1992). The consensus is:

$$I\ Q\ X_3\ R\ G\ X_{3\text{-}4}\ R$$

where X=any amino acid.
However, considerable variation is possible. In addition to heavy chain-light chain interactions the two light chains are in contact with each other. This interface is crucial in the regulation of scallop myosin (see 1.2.4) and may well be important in the kinetic cycles of all myosins.

1.1.4.4 The Myosin Super-Family.

Myosin is not restricted to muscle tissues. Myosins similar to those found in muscle are found in the cytoplasms of most animal cells (Sellars and Goodson, 1995) and references contained within. Their function appears to transport material along actin filaments within the cell. However, an increasing number of "unconventional" myosins are being identified (Cheney and Mooseker, 1992; Morgan, 1995; Sellars and Goodson, 1995). All share a region showing similarity to muscle myosin's (myosin II) motor domain and, consequently are actin activated MgATPases. The best characterised of these is myosin I (Pollard and Korn, 1973) - a single-headed molecule with no α-helical coiled-coil tail. Instead the C-terminus of the molecule binds phospholipids and the function of myosin I is thought to be the transport of membrane-bound vesicles along actin "tracks" within the cell. There are at least ten (Sellars and Goodson, 1995) classes

of unconventional myosins with at least one class being found in plants (Knight and Kendrick-Jones, 1993), showing that while muscles may be unique to the animal kingdom, myosins are not. Indeed "unconventional" may prove to be an unfortunate name for non-muscle myosins: many of them must predate muscle evolutionarily. It is estimated that the average animal cell expresses representatives of at least eight myosin classes.

1.1.4.5 The Molecular Motor Concept.

Myosin is a molecular motor. A typical motor protein has a globular head, comprising the motor domain. This domain hydrolyses nucleotide triphosphate to provide energy to fuel the motor and binds to a second protein - the "track" along which the motor runs (actin in the case of myosins). C-terminal to this domain is a neck region or regulatory domain. This region binds accessory proteins (myosin light chains in the case of myosin II, calmodulin in the case of many "unconventional" myosins) which can affect the activity of the motor domain (inferring communication between the two domains). The third, C-terminal domain - termed the cargo domain - shows the greatest variation. In myosin II it is the α-helical coiled-coil: the cargo is essentially another myosin molecule. In myosin I the cargo domain is the phospholipid binding region and the cargo any vesicle that is bound to it. Figure 1.5 shows the "consensus" design of this sort of molecular motor.

There are other molecular motors found in cells. The best characterised are the microtubule based motors found in most eukaryotic cells. Examples of this class include kinesin (and related proteins such as ncd) and dynein. Interestingly, the structure of the core of kinesin and a related protein (ncd) show great similarity to the core of myosin S1 suggesting that there may be similarities in the mechanism (Kull *et al.* 1996; Sablin *et al.* 1996). Other more diverse systems share the key feature of all these molecular motors: moving along a track in response to nucleotide triphosphate hydrolysis (eg RNA polymerases). Looking at these diverse systems as molecular motors provides a unifying concept and drives the search for common mechanistic features.

1.1.5 The Properties and Structure of Actin.

1.1.5.1 Properties of Actin.

Actin is a globular protein of molecular mass 42 kDa. It is found in most eukaryotic cells. The monomer readily self-associates to form filaments. A variety of conditions favour the filamentous form (F-actin) over the monomeric (G-actin): the concentration of actin must be over a certain critical level and the presence of physiological salt concentrations and/or MgATP will lower this critical concentration (see review by Sheterline *et al.* 1995). The actin filament consists of two stands of actin monomers twisting around each other. There are contacts between monomers along each strand and between strands (Holmes *et al.* 1990). Consequently, much of the surface of actin monomers is hidden in F-actin: only a limited area is available for the numerous interactions that actin undergoes. Actin interacts with many proteins which regulate filament assembly (eg gelsolin and profilin) and proteins which control the assembly of the cytoskeleton (eg dystrophin, spectrin) and with actin based molecular motors (eg the myosin super-family) (Sheterline *et al.* 1995 and references contained within). Most of the surface of the actin monomer is involved in some kind of interaction and it is therefore not surprising that the primary sequence of actin is remarkably well preserved through evolution (Sheterline *et al.* 1995).

1.1.5.2 Structure of Actin

The crystal structure of G-actin has been solved in complex with DNase-I (Kabsch *et al.* 1990). DNase-I forms an extremely stable (K_d is in the nmol range (Mannherz *et al.* 1980)) but presumably non-physiological (the proteins are found in different cellular compartments) complex with G-actin which prevents filament formation and traps actin in the monomeric state. The structure reveals a molecule with two domains separated

by a deep, nucleotide binding cleft (figure 1.6a). Each of the two domains is further divided into two sub-domains. Both the N- and C-termini are in sub-domain one.

With small structural rearrangements in the monomer, the model can be fitted into the electron density envelope produced by the (far lower resolution) electron-microscopy image reconstruction of F-actin and a model of the actin filament derived (Holmes *et al.* 1990) (figure 1.6b). This shows that, sub-domains three and four are primarily involved in actin-actin contacts within the filament whereas sub-domains one and two are more exposed. It is to be expected that these two sub-domains will contain the main sites of interaction between F-actin and other proteins. The main actin-actin contacts are in the long pitch of the helix rather than across the helix.

1.1.6 The ActoMyosin Complex

The Holy Grail of structural studies on motor proteins from muscle is to have models of the actomyosin complex at discrete points along the MgATPase cycle. This goal is a long way from being realised. The only atomic models of the actomyosin complex (Rayment *et al.* 1993a; Schroder *et al.* 1993) are derived from the model of F-actin (Holmes *et al.* 1990), the crystal structure of S1 (Rayment *et al.* 1993b) and the electron microscopy image reconstruction of acto-S1 (Milligan *et al.* 1990). The picture (figure 1.7) while valuable, should be treated with care. In order to fit S1 into the electron density envelope from the image reconstruction, major structural rearrangements had to be assumed in S1. Specifically, the cleft in the head of the molecule had to be closed. It is possible, but by no means certain that closure of this cleft is one of the principal steps in the MgATPase cycle. Even if it is then how this change is converted to longitudinal motion is not known. It should be noted that the light chains in this model are far from the sites of interaction with actin.

It is difficult to imagine how acto-S1 could be crystallised given actin's tendency to polymerise. One possible solution may be the generation of intra-molecular cross-linked actin monomers which remain monomeric but behave like "F-actin monomers" (Heintz and Faulstich, 1996).

1.1.7 The MgATPase Cycle and Energy Transduction.

The actin-activated MgATPase cycle of myosin has been studied extensively. Unfortunately, these studies have not reached any particular consensus as to the fine detail of the cycle. What is agreed is that the two proteins undergo cyclic attachment and detachment from one another. During the so-called power stroke, the myosin head undergoes a dramatic conformational change which results in the movement of the filaments relative to one another. Furthermore, the energy released by MgATP hydrolysis is not utilised directly (indeed the equilibrium constant for the reaction: MgATP + $H_2O \leftrightarrow$ MgADP + P_i in the active site of myosin is close to one). One popular (but by no means necessarily correct as a result) kinetic model is that of Eisenberg and Greene (Eisenberg and Greene, 1980) and reviewed by Eisenberg and Hill, 1985. This model (figure 1.8) incorporates the results of kinetic studies *in vitro* along with ideas about how myosin and actin are organised in muscle tissue and it provides a plausible mechanism by which a kinetic cycle can result in unidirectional motion.

The model proposes that actomyosin can exist in two states: a "strongly" attached state in which the rate of dissociation of myosin heads from actin is very low and a "weakly" attached state in which actin + myosin head are in rapid equilibrium. The binding of MgATP to the myosin head triggers the switch from the strongly to the weakly attached state (I in (figure 1.8)). Once bound to myosin, MgATP undergoes hydrolysis to MgADP and P_i (II) but the products remain bound (and are in equilibrium with the starting material). Furthermore, the hydrolysis of MgATP does not result, directly, in any great structural changes in the myosin head. Structural changes (detected by fluorescence changes in S1 (Marsh *et al.* 1982)) do occur as myosin with ADP and P_i bound undergoes isomerisation to a form which is primed to release P_i (III). This isomerisation is the rate determining step of the Eisenberg and Greene model (Eisenberg and Greene, 1980).

Phosphate release results in a return to the strongly attached state (IV). In doing so, the myosin head undergoes dramatic structural changes altering its approximate angle of interaction with actin from 90° in the weakly attached state to 45° (detected by X-ray fibre diffraction (Peckham and Irving, 1989) and fluorescence studies (Irving *et al.* 1995) of muscle fibres undergoing synchronised contraction). However, because the actin binding sites on the myosin heads (and their complementary sites on actin) are constrained in space by the lattice-like nature of the thick and thin filaments, this state is strained and, consequently, force is developed at this point. This stain is relieved by relative motion of the actin and myosin filaments (V). The force is dissipated and work is done, resulting in a stable 45°, MgADP bound, strongly attached state.

Exchange of bound MgADP for MgATP (VI) returns the system to the start: a 90°, weakly attached state. Like the 45° state produced by P_i release, this state is a strained state (again due to constraints resulting from the arrangement of the filaments *in vivo*). In this case, however, the strain is not relieved by motion of the actin and myosin filaments (if it did it would return them to their original positions) but by the nature of the interaction with actin. The weak binding of the myosin head to actin means that the strain can be relieved while the head is "free" and not constrained by interaction with actin. It is this key stage in the cycle which permits a repeating cycle to result in directional linear motion.

The cycle works as a motor transforming chemical energy into mechanical energy not because the free energy of MgATP hydrolysis is stored directly in an energised form of myosin, but because the binding of MgATP is used to control the interaction of myosin with actin. Although MgADP and P_i are favoured energetically over MgATP in the sarcoplasm (because of differences in the chemical structure between ATP and ADP and the greater concentration of MgATP), the two forms are in stable equilibrium in the catalytic site of myosin. The binding of MgATP (or MgADP + P_i) by providing new interactions for myosin, compensates for the loss in free energy resulting from the (partial) dissociation from actin. However, the release of MgADP and P_i from the myosin head is favoured energetically (unlike the release of MgATP) and the release of P_i along with the return to high affinity binding for actin provides the free energy for

movement. The purpose of MgATP hydrolysis is to provide directionality to the process, by making it thermodynamically irreversible (Eisenberg and Hill, 1985).

The cycle has been interpreted taking recent structural data on the actomyosin complex into account (Reedy, 1993; Spudich, 1994; Rayment and Holden, 1994; Ruppel and Spudich, 1995; Cooke, 1993). The transition between weakly and strongly attached states is presumed to involve the recruitment of additional binding sites for actin on the myosin head, corresponding (possibly) to closure of the cleft seen in the S1 structure which had to be closed to fit this structure into the electron microscopy image reconstruction models of acto-S1 (Rayment *et al.* 1993a; Cooke, 1993). However, the fine details of the structural changes (and the assignment of these changes to specific steps in the cycle) along with details of which contact sites are important at each stage will have to wait until further structural information is available.

One unresolved question is how relatively small changes occurring on P_i release are amplified to produce a movement of between 0.8 and 1.5 nm. (Estimates for the "step size" - ie the distance moved per myosin head on MgATP hydrolysis vary roughly between these two extremes. From a structural point of view, the answer should be an integral multiple of the distance between two actin monomers, but proponents of other values defend them vigorously).

A common conjecture is that the light chain binding domain functions as a "lever arm" magnifying small structural changes in the motor domain (Rayment *et al.* 1993a; Trayer, 1993). If this is so, then large changes in the angle that the light chain binding domain makes with the actin filament should be seen through the cycle. By placing a fluorescent probe on the RLC in intact muscle fibres and then observing changes in the fluorescence polarisation during synchronised contractions, it was shown that this region of the myosin molecule does undergo large movements (Irving *et al.* 1995). Conversely, paramagnetic probes in the motor domain detected very little movement (Zhao *et al.* 1995). Electron microscopy image reconstruction shows a large movement of the tail domain in brush border myosin I (Jontes *et al.* 1995) and smooth muscle myosin II (Whittaker *et al.* 1995) on ADP release. However the most striking evidence

for the lever arm hypothesis comes from *in vitro* motility assays with myosins (and myosin fragments) of altered arm length. Reduction of the length of the arm reduces the actin sliding velocity without altering the rate of MgATP hydrolysis (Lowey *et al.* 1993a) suggesting that it is the length of the arm that controls the sliding velocity and not the rate of MgATP hydrolysis. A particularly elegant study has brought the power of recombinant DNA technology to bear on the problem. Using the slime mould *Dictyostelium discodium* as an expression system, myosins of varying arm length were produced (Uyeda *et al.* 1996) - including one which had an additional ELC binding site. Sliding velocity *in vitro* correlated linearly with arm length.

1.2 The Regulation of Muscle Contraction.

1.2.1 Introduction: The Importance of Calcium Ions.

All muscle contraction is regulated by the intracellular free calcium ion concentration, but the means by which this signal is sensed and used as a trigger for contraction (or relaxation) varies with muscle type and source.

Following nervous stimulation, the sarcoplasmic membrane rapidly depolarises. This induces the influx of calcium ions into the sarcoplasm. This rise in calcium ion concentration results in calcium induced calcium release from the sarcoplasmic reticulum where vast reserves of calcium are stored. The process is extremely rapid; a hundred-fold calcium ion concentration increase (from 10^{-7} to 10^{-5} M) is achieved over a millisecond timescale. Of course, it is just as important to switch the muscle off again. Following stimulation, calcium ions are actively transported across the sarcoplasmic and sarcoplasmic reticulum membranes so that the free calcium ion concentration falls to resting levels.

1.2.2 Regulation in striated muscle: troponin/tropomyosin based signalling.

Vertebrate skeletal and cardiac muscle regulation depends on a protein switch based on four components: tropomyosin and the troponins C, T and I. Troponin C is the calcium

sensor. It is structurally similar to calmodulin (Herzberg and James, 1985; Findlay and Sykes, 1993; Gagne *et al.* 1995; Slupsky and Sykes, 1995) and binds two calcium ions per molecule (one in cardiac TnC) under activating calcium concentrations. The binding of calcium ions by TnC strengthens its interactions with TnI and TnT. In the off-state, TnI interacts with actin and inhibits the actomyosin MgATPase: the means by which it does this is not known. Tropomyosin is a dimer - consisting mainly of an α-helical coiled-coil (White *et al.* 1987; Whitby *et al.* 1992). It lies along the groove in the actin filament (figure 1.9) and probably blocks the interaction (or perhaps one of the interactions) between actin and the myosin head.

In order to switch on the muscle, Ca^{2+}-TnC must simultaneously relieve the TnI mediated inhibition and then cause the movement of tropomyosin to allow full actin-myosin interaction. Inhibition by TnI is removed directly: the strong binding of Ca^{2+}-TnC to TnI must promote structural changes in TnI (nothing is known about the structure of TnI) making it incapable of inhibiting. The effect on tropomyosin is mediated by TnT. The altered interaction of TnT with Ca^{2+}-TnC affects TnT's interactions with tropomyosin such as to promote a shift in the position of tropomyosin (which can be observed by X-ray fibre diffraction studies (Squire *et al.* 1993; Popp and Maéda, 1993; Ishiwata and Wakabayashi, 1994)) which removes the steric inhibition of actin-myosin interaction.

The troponin/tropomyosin switch is a complex, concerted mechanism subject to numerous subtle modifications. This area has been the subject of a number of recent reviews (eg Farah and Reinach, 1995; Tobacman, 1996).

Insects are unique amoung invertebrates in having a troponin-tropomyosin based system for regulating muscle contraction. However, the same differences are seen in the components of the troponin-tropomyosin complex: all three forms of troponin found in vertebrate muscle are present, along with a fourth form, TnH (80 kDa, H for heavy) which substitutes for tropomyosin. Antibodies raised against tropomyosin will also react with TnH. However, TnH also contains a unique sequence which is rich in hydrophobic and proline residues (Peckham *et al.* 1992). The function of this region is

not known. Interestingly, as well as being the only invertebrate group possessing the troponin complex, insects also have a light chain which may act in a similar way to A1-type ELCs of vertebrate striated muscle (see Chapter 6).

1.2.3 Regulation in Smooth Muscle: Calcium-Dependant Phosphorylation of the RLC

Smooth muscle myosin is regulated (principally) by phosphorylation of the RLC by a dedicated kinase myosin light chain kinase (MLCK). This phosphorylation event has two effects: (i) the actin-activated MgATPase of myosin is switched on and (ii) the myosin molecule undergoes a dramatic conformational change from a compact form (with a sedimentation coefficient of 10S) to an elongated (6S) form which can form filaments (reviewed by Jiang and Stephens, 1994; Allen and Walsh, 1994). The phosphorylation is reversed by the action of protein phosphatase I, targeted to myosin by the M110 targeting sub-unit (Johnson *et al.* 1996).

The RLC of smooth muscle has been the target of intensive mutagenic studies. Neither the N- or C-terminal residues are resolved in the crystal structure of skeletal S1 (Rayment *et al.* 1993b), suggesting that theses residues are disordered. Deleting residues at the N-terminus shifts the equilibrium between the two folding states of myosin to the 6S form (Ikebe *et al.* 1994c) (residues Lys 11 to Arg 16 seem to be the most important). However, this 6S form while capable of forming filaments, still requires phosphorylation for activation of the MgATPase. This suggests that the two functions of phosphorylation of the wild type RLC are not directly coupled (as one can occur without the other).

Similar deletion studies have established what is (and what is not) important in the recognition of the RLC by MLCK. Deletion of the first ten residues has little effect (Ikebe *et al.* 1994b). Critical residues are Arg 16 and some hydrophobic residues C-terminal to the phosphorylatable serine (Ser 19) (Ikebe *et al.* 1994b; Zhi *et al.* 1994). However, the N-terminal residues alone are not enough. Synthetic peptides corresponding to these residues are phosphorylated at much reduced rates as are

chimeric RLCs in which sub-domains one and two are substituted for their skeletal equivalents (Zhi *et al.* 1994). This suggests that in addition to interacting with residues immediately surrounding the phosphorylation site, the kinase also recognises sites in sub-domains one and two.

A computer generated model of the N-terminus of the RLC (Ikebe *et al.* 1994b) places the guanidino group of Arg 16 close to the phosphorylation site. This could explain why this residue is so crucial in the phosphorylation reaction: its rôle could be to stabilise the transfer of the negatively charged phosphate group onto the protein. However, this model (which is based on no experimental evidence) should be treated with extreme caution, not least because it is only a model of the N-terminal peptide. This peptide shows a different reactivity to MLCK than the whole protein does and this may well be because sub-domains one and two influence the folding of the extreme N-terminus.

The C-terminal residues are also important in phosphorylation dependent regulation. Deletion of the last twenty six residues of the RLC and exchange of this mutant for wild type results in a myosin which behaves as if the RLC were totally absent (except that it does not aggregate) (Trybus *et al.* 1994): even on phosphorylation these myosins exhibit only a basal level of activity. Results from this study and a similar one (Ikebe *et al.* 1994a) suggest that the C-terminus is required for transmission of the structural change resulting from phosphorylation to the active site.

Only two headed forms of smooth muscle myosin are regulated by phosphorylation: HMM and whole myosin are; S1 is constituitively active. S1 lacks the potential for head to head interaction and much of the flexible "joint" between the myosin head and the rod-like tail. The two RLCs in HMM can be cross-linked (using 5,5′-dithiobis(2-nitrobenzoic acid)) *via* their cysteine thiols (Katoh and Morita, 1995) suggesting that they are close in space. This reaction does not occur in skeletal muscle myosin. A single headed species produced by proteolysis is unregulated (Cremo, R. *et al.* 1995). An elegant protein engineering study confirmed the requirement for a two-headed structure. A series of N-terminal deletion fragments of smooth muscle myosin were

expressed in a baculovirus expression system beginning with a construct that resembles S1 and ending with one similar to HMM. When more than 991 residues were included the structure was double-headed (as judged by electron microscopy and gel filtration chromatography) and their MgATPase activity is regulated by phosphorylation. Constructs shorter than 941 residues were single headed and unregulated (Matsu-ura and Ikebe, 1995). The authors postulated that phosphorylation of the RLC results in structural changes in the head/rod junction which in turn lead to disruption of the head-head interaction and switching on of the myosin motor.

Monoclonal antibodies to the ELC inhibit RLC exchange in HMM and myosin, but not S1 (Higashihara and Ikebe, 1995) - again suggesting the importance of a two-headed structure. Furthermore, this suggests that the RLC/ELC interface seen in skeletal S1 (Rayment *et al.* 1993b) and the scallop regulatory domain (Xie *et al.* 1994; Houdusse and Cohen, 1996) also exists in smooth muscle myosin and that it may have a rôle in information transfer from the RLC to the active site. Another study casts doubt on this interpretation (Trybus, 1994b). HMM with intact smooth muscle RLC (smRLC) and smELC exhibits phosphorylation dependent regulation. If the smELC is swapped for its skeletal homologue there is no change in the regulation of the resulting myosin. Although regulation is insensitive to ELC source, it is abolished by changing the RLC for its skeletal counterpart: this HMM is always switched off. Most importantly, HMM containing no ELC (but with smRLC) is regulated by phosphorylation - although the MgATPase activity is about one quarter of the native form. Since the message can pass to the active site in the absence of the ELC, this rules out the ELC/RLC interface as the sole route of communication between the RLC and the active site.

Many of the uncertainties surrounding the phosphorylation dependant activation of smooth muscle myosin would be removed by a clear, structural model for the changes occurring in the RLC and the heavy chain segment it encloses on phosphorylation. One group of workers has already begun this task by developing methods to form and purify RLC/heavy chain peptide complexes *in vitro* (Ferguson *et al.* 1995). However, the problem is made more difficult by the RLC's tendency to self-aggregate at the concentrations required for serious NMR analysis (Dingley *et al.* 1995).

Phosphorylation of the RLC is not the only control on smooth muscle contraction. Three thin filament associated proteins also play a part: tropomyosin, calponin and caldesmon (Allen and Walsh, 1994; Jiang and Stephens, 1994; Walsh, 1994). Their precise functions are uncertain. Calponin and tropomyosin are present at the strict stoichiometry of one tropomyosin to one calponin to seven actin monomers whereas caldesmon is present at varying amounts depending on the tissue. Calponin interacts with tropomyosin and also inhibits the MgATPase of smooth muscle myosin. This inhibition is relieved by the phosphorylation of calponin. This phosphorylation is activated by calcium ions, but higher concentrations are required than for the activation of calmodulin/MLCK. Calponin may act in a similar way to TnT, by controlling the position of tropomyosin on the actin filament; its function may be to provide a range of different activities of smooth muscle myosin - enabling the system to respond to different conditions. Caldesmon binds to actin and inhibits the MgATPase of smooth muscle myosin (possibly in a way analogous to TnI). This inhibition is relieved by (among other events) binding to Ca^{2+}-calmodulin.

The complex nature of smooth muscle regulation has prompted a number of authors to develop computer models (Haeberle and Hemric, 1994; Slawnych *et al.* 1994). It should be emphasised that the principal trigger for activation is a single, covalent change: the introduction of a phosphate group at position 18 of the RLC. This relatively small change results in profound changes in both the structures and enzymatic activity of smooth muscle myosin. One of the most important questions to ask is how the consequences of this change are transmitted through the molecule.

1.2.4 Regulation of Scallop Myosin: Direct binding by Ca^{2+} activates the motor.

The rôle of calcium ions in the direct regulation of scallop myosin has been known for some time (Szent-Györgyi *et al.* 1973). However, in one of the true triumphs of modern structural biology, the location of the binding site for this regulatory ion had to wait for the solution of the crystal structure of the regulatory domain of scallop myosin (Xie *et al.* 1994; Houdusse and Cohen, 1996). Indeed this structure (figure 1.10) throws new

light on previous studies on isolated proteins and permits rational protein engineering experiments to be undertaken.

Like smooth muscle myosin, the scallop molecule is only regulated when both heads are present: S1 is constituitively active whereas HMM is calcium sensitive (Szent-Györgyi *et al.* 1973) - suggesting that, as in smooth muscle myosin, head-head interactions are important. Loss of the RLC desensitises HMM (and leads to aggregation of the molecule - presumably because a length of the 8 nm, hydrophobic α-helix is exposed), but does not reduce calcium binding to zero (Szent-Györgyi *et al.* 1973). Replacing the RLC with skeletal RLC prevents aggregation and switches the myosin off but does not restore calcium sensitivity. Interestingly, smooth muscle RLC restores Ca^{2+}-sensitivity - suggesting that RLCs from myosins that are directly regulated (as opposed to those that are regulated *via* the thin filament) share common features and that the pathway of information transfer may be similar in both classes of myosin.

The crystal structure of the scallop regulatory domain (Xie *et al.* 1994) shows that the calcium ion binding site is in the ELC. However, this calcium binding site is unlike any other known EF-hand binding site, and has a sequence that would not have been predicted as a functional calcium binding site by comparison to other known sites. The ion is co-ordinated by seven interactions in a pentagonal bipyramidial configuration, including three from back-bone carbonyl groups and three from aspartate side chains. The structure has been refined recently (Houdusse and Cohen, 1996), and the nature of this co-ordination pattern confirmed - along with the rôle of a tightly bound water molecule filling the remaining co-ordination position. The site exists only because the ELC is constrained by interactions with the heavy chain and, especially, with the RLC: full calcium binding is only observed when all three proteins are present and the free ELC has no calcium binding activity. The nature of the structural changes induced by the binding of calcium can only be guessed at (Houdusse and Cohen, 1996) in the absence of a structure lacking a bound calcium ion

The structure highlights the importance of the RLC/ELC contact which is mediated by a network of hydrogen bonds. These interactions play a major part in constraining the

calcium-binding site in the ELC. Gly 117 is one of the residues involved in hydrogen bonding to the ELC and its critical rôle is confirmed by mutagenesis experiments. Chimeric RLCs show that only domain one (the one that contacts the ELC) is required to restore full calcium sensitivity to the scallop system: a chimera composed of scallop domain one and the remainder of the sequence from rabbit skeletal RLC behaves as wild type (Fromherz and Szent-Györgyi, 1995). Furthermore, mutations made in residues 81-117 of scallop RLC show that only two have any effect Gly117 to Cys and Gly117 to Ala (Jancso and Szent-Györgyi, 1994). Both affect calcium binding and sensitivity and reduce the affinity of the RLC for the heavy chain. If the equivalent position in the rabbit skeletal sequence (Cys in the wild type) is changed to Gly then calcium activation and regulation is restored (Jancso and Szent-Györgyi, 1994).

1.3 Long Term Control of muscle Contractility.

Although calcium ions are the universal trigger for muscle contraction, other events can fine tune this response on a longer time scale. All muscles respond to elevated calcium ion levels, but the way in which they do depends on how the regulatory proteins and the contractile proteins themselves behave after this signal has been detected.

Clearly, the response can be modulated by altering the proteins involved in the signalling cascade or contraction. In the medium term, this may be effected by reversible, covalent modification of particular proteins (eg phosphorylation of TnI in cardiac muscle appears to reduce calcium sensitivity (Al-Hillawi *et al.* 1995)). In the longer term, altering the expression of these proteins for alternative isoforms provides a way for muscles to adapt over this time-scale. Furthermore, the expression of tissue-specific isoforms means that different muscle types will have different properties and different responses to calcium activation.

1.3.1 Myosin isoform diversity in mammalian muscle.

Three types of muscle may be distinguished histologically in mammals: smooth, cardiac and skeletal; the latter two show considerable similarity and are often considered

together as striated muscle. The three types may be distinguished on the basis of their contractile properties and - to a large part - this is due to their myosin isoform content. Each has its own, specific group of myosin heavy and light chain isoforms.

Skeletal fibres may be further sub-divided into two categories: fast and slow, again with characteristic myosin isoforms and, consequently, contractile properties. In rat, rabbit and human slow skeletal muscle only one heavy chain and one ELC isoform are known. In contrast three forms of the heavy chain have been identified in rat fast skeletal muscle termed A, B and D (although only two, A and D are known in humans) on the basis of sensitive gel electrophoresis and immunochemistry (reviewed by Sellars and Goodson, 1995). Furthermore, two ELC isoforms are known in this tissue: A1 (or LC1) and A2 (or LC3). In slow skeletal, atrial and ventricular muscle only A1-type ELCs are found. Smooth muscle contains only A2-like ELCs.

1.3.2 Myosin Heavy Chain Diversity and its Effect on Muscle Contraction.

Modern advances in electrophoresis and the uses of isoform specific monoclonal antibodies have made possible the identification of myosin heavy chain isoforms even though the proteins differ only slightly. The isoforms expressed in rat skeletal muscle are summarised below (after Schiaffino and Reggiani, 1994).

	Isoforms expressed mainly in:		
Gene Family	Developing muscles	Fast muscles (type I fibres)	Slow muscles (type II fibres)
MHC (myosin heavy chain)	MHC-emb MHC-neo	MHC-IIa MHC-IIb MHC-IIx	MHC-I
MLC1 (ELC)	MLC-1emb [(1), (2)] MLC-1f [(2)]	MLC-1f [(2)] MLC-3f [(3)]	MLC-1s [(2)]
MLC2 (RLC)	MLC-2f	MLC-2f	MLC-2s

[(1)] This protein is also expressed in atrial muscle.

[2] A1-type

[3] A2-type

Interestingly, there are three heavy chain isoforms found in fast skeletal muscle. That these isoforms result in different contractile properties was confirmed by elegant experiments in which fibres containing only one isoform were isolated (most fast skeletal fibres contain a mixture of types (Sweeney *et al.* 1988)). The myosin heavy chain type correlates well with the unloaded shortening velocity (V_0) of the fibre. Myosin heavy chain type I (MHC I) is much slower than any type II isoform (Galler *et al.* 1994). Of the type II isoforms, IIA is the slowest, IIX intermediate and IIB the fastest (Bottinelli *et al.* 1994a; Schiaffino and Reggiani, 1994; Galler *et al.* 1994). The MgATPase activity also correlates to the MHC isoform with again, MHC IIA having the lowest MgATPase activity and MHC IIB the highest (Bottinelli *et al.* 1994b). Thus, it seems likely that (at least) three MHC types exist to provide a variety of contractile speed among fast fibres. That single fibres often contain a variety of types, suggests that, by mixing the isoforms, a range of fibre properties can be produced.

The isoform composition of a particular muscle is not static. External influences (eg drugs, exercise, electrical stimulation) can alter the pattern of expression of myosin isoforms. The effects of exposure to zero gravity during space-flight have been well studied. Rats sent into orbit for relatively short periods (around one week) show measurable changes in MHC type. Specifically, they showed a shift towards "faster" isoforms (Caiozzo *et al.* 1994). Levels of MHC I decreased and MHC II increased. However, MHC IIA (the slowest of the fast isoforms (Bottinelli *et al.* 1994a)) also decreased relative to MHC IIB and IIX. Fast skeletal specific ELC and RLC isoforms also increased. These changes result (at least in part) from changes in gene expression (rather than selective degradation of some isoforms): mRNA levels were also seen to change in a way which corresponded to the alterations in amounts of protein (Esser and Hardeman, 1995). The effect is also seen in human subjects (Zhou *et al.* 1995) - although considerable variation between individuals is also seen.

Thus it appears that the variety of myosin heavy chain isoforms represents the means by which muscles can adapt to different demands. Furthermore, this adaptation can be controlled in the longer term by changing the expression of genes for the various isoforms. How the wide variety of environmental stimuli are processed into a co-ordinated message which is interpreted at the level of gene expression is an important and challenging question which largely remains unanswered.

1.3.3 Myosin Essential Light Chains: Fine Tuners of the Heavy Chain?

Initial attempts to relate ELC content of muscle fibres to their contractile properties were confused by the existence of fibres of mixed myosin heavy chain type and the preferential association of ELC isoforms with particular heavy chain types (Bottinelli *et al.* 1994b) (A1 with MHC IIA and A2 with MHC IIB). The problem was overcome by Bottinelli and co-workers (Bottinelli *et al.* 1994a) in their studies on fibres of a single heavy chain type. They detected a significant correlation between the ELC isoform and V_0 in all three fast skeletal isoforms - the effect being greatest in MHC IIA fibres. V_0 is proportional to the amount of A2-type ELC (MLC 3) in the fibre - suggesting that A1-type ELCs (MLC 1) tend to slow down their heavy chain whereas A2 tends to speed it up. However, the ELC isoform has no effect on the MgATPase activity of myosins in muscle fibres (Bottinelli *et al.* 1994b), suggesting that MgATP turnover is not directly related to the speed of movement.

The results from whole muscle fibres agree with those from less physiological systems: HMM in *in vitro* motility assays moves actin at a speed which depends on the ELC isoform it is carrying (Stepkowski *et al.* 1994); S1 carrying A1-type ELC (S1(A1)) is slower at hydrolysing MgATP in solution than its isoenzyme (S1(A2)) (Weeds and Taylor, 1975).

Since myosin heavy chain isoform is the greatest determinant of V_0 (Bottinelli *et al.* 1994a; Schiaffino and Reggiani, 1994; Moss *et al.* 1995) and other physiological

properties of muscle fibres (Moss *et al.* 1995), it seems that the rôle of the ELC is to fine tune the heavy chain permitting a continuum of contractile properties from any one heavy chain isoform (Schiaffino and Reggiani, 1994). Therefore, it would be expected that ELC types (A1 *vs* A2) would be subject to changes in levels of expression resulting from external stimuli. Indeed since the phenotypic changes resulting from such alterations would be more subtle than those resulting from changes in MHC gene expression, it might be expected that lower levels of stimulus should bring them about.

1.3.4 The Regulatory Light Chains: Molecular Memory of Past Activity?

The rôle of the RLC in controlling smooth muscle contraction by reversible phosphorylation is well established (see section 1.2.3). However, the RLC of skeletal muscle can also be phosphorylated by a specific Ca^{2+}-dependant kinase (and dephosphorylated by a phosphatase targeted by a specific sub-unit). Although not involved in the regulation of skeletal muscle contraction, it seems unlikely that such a system would have been retained though evolution if it served no purpose. Most work on the phosphorylation of the skeletal muscle RLC suggest that it serves as a "molecular memory" of past activity.

Phosphorylation of the RLC results in potentiation of contractile activity: the rate of force development is increased, tension is increased and the tension *vs* $[Ca^{2+}]$ curve shifts to the left (Bottinelli *et al.* 1994b) (ie more force is generated at lower calcium concentrations when the RLC is phosphorylated). Another study shows that calcium sensitivity is decreased on phosphorylation (Vandenbroom *et al.* 1995).

One possible explanation for this is that repeated stimulation by elevated calcium concentrations, not only results in repeated contraction but also in Ca^{2+}-calmodulin dependant phosphorylation of the RLC. This in turn leads to deceased calcium sensitivity of the contractile system, resulting in a myosin which responds more rapidly to an influx of calcium ions (because it responds at a lower concentration). This would have at least two potential benefits: the interval between contractions would be reduced (assuming that this interval is limited by the rate of calcium influx and efflux) and

energy would be saved as less calcium would need to be pumped. Furthermore, if the phosphorylated state persisted after the cessation of contraction, it would serve as a memory of recent contraction - permitting a rapid response to renewed activity (Stepkowski, 1995).

The molecular mechanisms by which RLC phosphorylation results in decreased calcium sensitivity is not known. The crystal structure offers no clues: the phosphorylatable residue (Ser 15) is not resolved (Rayment *et al.* 1993b), suggesting that they are disordered. It is also likely that the S1 used for crystallisation contained a mixture of phosphorylated and dephosphorylated protein. Given the arrangement of the proteins in the myofilament it is difficult to imagine any direct interaction between the RLC and the calcium sensor TnC. This suggests that any signal must pass from the RLC to the heavy chain - perhaps *via* the ELC which it is in contact with (Rayment *et al.* 1993b) (conformational changes in the RLC do affect the ELC (Stepkowski *et al.* 1994)). One possible explanation is that phosphorylation of the RLC increases the affinity of the heavy chain for actin - which would affect the equilibrium between the "blocking" and "non-blocking" positions of tropomyosin, resulting in (effectively) decreased calcium sensitivity.

1.4 Biochemical studies on Myosin Isoenzymes.

1.4.1 Myosin as an Enzyme.

Myosin's enzymatic activity is to hydrolyse MgATP to MgADP and P_i. In the absence of actin the activity is very low but this is activated many-fold in its presence. Thus the MgATPase activity of myosin (and its proteolytic fragments) increases with increasing actin concentration. This increase is not linear; it shows saturatable kinetics similar to that seen by Michaelis-Menton enzymes on increasing substrate concentration. It is, therefore, possible to fit activity (normalised for active site concentration) *vs* [actin] curves to the Michaelis-Menton equation (Cornish-Bowden, 1979) in order to derive two constants: an apparent K_M for actin (which will be the actin concentration which gives half-maximal MgATPase rate) and k_{cat} (which will be the number of molecules of

MgATP turned over per active site per second). Both constants are important: K_M tells us about the interaction between myosin and actin and k_{cat} tells us how fast the motor is working.

For most practical kinetic work on myosin, S1 is used. This fragment contains only a single active site and (unlike myosin) is soluble over a wide range of ionic strengths.

1.4.2 Differences Between A1 and A2 at the Level of Primary Structure.

There are two ELC isoforms in fast skeletal muscle: A1 and A2. For much of their sequence they are identical (Frank and Weeds, 1974); they differ only at their N-termini where A1 has 42 (in rabbit skeletal muscle) additional amino acids. There follows 8 residues of high sequence similarity and then 140 identical residues. The two proteins result from differential mRNA splicing of the same gene transcript (Nabeshima *et al.* 1984) (figure 1.11). Since the two proteins are similar, or identical along much of their length, it seems reasonable to assume that any functional differences between the two will result from these additional N-terminal amino acid residues in A1. Indeed, to ask what is the function of this extension is equivalent to asking what the functional differences between the two isoforms are. Figure 1.12 shows the sequences of rabbit skeletal A1 and A2.

The N-terminal amino acid residue of A1 and A2 is blocked. The blocking group in A1-type light chains - trimethylalanine - is not commonly found; its only reported occurrence in vertebrate systems is in myosin light chains (Henry *et al.* 1982; Henry *et al.* 1985a). The formation of quaternary ammonium groups is energetically expensive and therefore some adaptive advantage would be expected from it. The nature of this advantage is not known. The first ten or so amino acids of A1 contain a high proportion of lysine residues, giving this region a positive charge (which contrasts with the molecule as a whole which has an acidic isoelectric point). The remaining 30-odd residues of the extension is rich in proline residues and much of the sequence consists of the repeat (Xxx-Pro) where Xxx is often alanine.

1.4.3 Kinetic Properties of S1 Isoenzymes

The existence of two ELC isoforms in fast skeletal muscle gives rise to the possibility of two S1 isoenzymes - S1(A1) and S1(A2) - depending on which ELC type is carried by the heavy chain. The two isoenzymes can be separated (Weeds and Taylor, 1975) and at low ionic strength (< 10 mM salt) have different actin activated MgATPase kinetics. Under these conditions, S1(A1) has a 5-fold lower K_M for actin and a 2-fold lower k_{cat} than S1(A2). This suggests that S1(A1) has a higher affinity for actin, but turns over more slowly. These results are only seen in the presence of actin: k_{cat}'s for S1(A1) and S1(A2) in its absence are identical (Weeds and Taylor, 1975).

These results are borne out when ELCs from a variety of sources were tested (Wagner and Weeds, 1977). These investigators developed a method for exchanging one ELC in S1 for another - resulting in "hybrid" S1s. Their results show that, against a common heavy chain background, the kinetic properties of S1 depend on the ELC and that there are two classes of ELC: A1-like and A2-like. All A1-like (ie possessing the N-terminal extension) ELCs confer similar properties on S1 regardless of their source - as do all A2-like ELCs. This study also shows that it is possible to exchange the ELC (the method involves the mild denaturation of S1 with ammonium chloride) without loss of activity.

The rates in these two studies were determined by measuring proton release on MgATP hydrolysis in a pH-stat. Necessarily they were carried out with low buffer concentrations and also at low ionic strength. When the experiments were repeated with myosin (rather than S1) and at higher ionic strength (Pope *et al.* 1981) two interesting results were seen. First there was no longer a simple dependence of the rate on actin concentration - two distinct phases were seen and second there was no dependence on the ELC content. Clearly myosin is a more complex system than S1 (which only shows a single phase of actin activated MgATPase activity). Furthermore, it is not entirely unexpected that the differences between S1(A1) and S1(A2) become increasingly difficult to detect (Bottomley and Trayer, 1975; Wagner *et al.* 1979) as the activity of S1 is itself much reduced at physiological ionic strength.

1.4.4 Other Biochemical Differences Between Myosin Isoenzymes.

1.4.4.1 In Vitro *Motility Assays.*

This system measures the forces and velocities between motor proteins and their partners. In the most common arrangement, myosin (or one of its fragments) is immobilised onto a solid support and F-actin introduced above it. The actin filament is stabilised (usually with phallodin - a fungal toxin which inhibits depolymerisation (Sheterline *et al.* 1995)) and visualised by labelling with a fluorescent probe. When viewed under a fluorescence microscope, the actin filaments are seen to move as they interact with the immobilised myosin. The velocity of movement can then be determined by computer analysis of video images of moving filaments. Alternative arrangements are used for measuring force: the moving actin filament can be attached to very fine glass needles and the force calculated from the deflection (Kishino and Yanagida, 1988) or sophisticated optical trapping methods can be used (Nishizaka *et al.* 1995).

Lowey and co-workers (Lowey *et al.* 1993b) prepared myosins enriched in each of the ELC isoforms using monoclonal antibodies specific to the unique regions of the ELCs (the extension on A1 and the region of similarity - rather than identity - on A2). The two populations moved actin in an *in vitro* motility assay at significantly different velocities (Lowey *et al.* 1993b) with the A1-enriched population moving actin more slowly than the A2-enriched one.

1.4.4.2 Studies on whole muscle fibres.

The results from single muscle fibre experiments using fibres with only a single heavy chain isoform present have already been dealt with in section 1.3.3. It should be noted

that all three methods of studying myosin isoenzymes (MgATPase kinetics, *in vitro* motility assays and single fibre studies) are in general agreement about the differences between the two forms: myosins carrying A1-type ELCs are - all other things being equal - "slower" than those carrying A2-like ELCs.

1.4.5 Binding of myosin fragments and the ELC isoforms to actin.

1.4.5.1 Differential binding of S1 and HMM isoenzymes to F-actin.

The lower apparent K_M for actin of S1(A1) (Weeds and Taylor, 1975; Wagner and Weeds, 1977; Winstanley *et al.* 1979) suggests that this isoenzyme may interact more strongly with actin. This proposition is supported by cross-linked F-actin-tropomyosin affinity chromatography experiments. In low ionic strength (5 mM TEA.HCl) buffer. S1(A1) is bound more strongly by the column than S1(A2) and required salt (0.25 M KCl) to elute it. The two isoenzymes are not resolved at higher ionic strengths (Bottomley and Trayer, 1975). In the low ionic strength buffer, HMM can be fractionated into HMM(A2,A2) homodimers, HMM(A1,A2) heterodimers and HMM(A1,A1) homodimers with the latter eluting at the highest salt concentration (Trayer *et al.* 1977).

It is of concern that no difference (either kinetic or in binding) between the isoenzymes was detected at ionic strengths even approaching physiological (≅ 150 mM NaCl). However, S1 is itself not a physiological system and the acto-myosin system interacts with a number of other proteins *in vivo*. Furthermore, S1's (either isoenzyme) MgATPase activity is greatly reduced by increasing ionic strength (Bottomley and Trayer, 1975) and most studies (even those not seeking to detect a difference between S1(A1) and S1(A2)) have been carried out in ionic strengths considerably less than 150 mM.

The only serious study of the binding properties of S1(A1) and S1(A2) to regulated actin (ie in the presence of troponin-tropomyosin) at different calcium concentrations and in the presence of different nucleotides (Trayer and Trayer, 1985) did detect a

difference. Indeed with MgADP as the nucleotide, in the presence of activating concentrations of calcium ions, S1(A1) binds more strongly to actin (as detected by co-sedimentation assays) than S1(A2) even at an ionic strength of 270 mM. This suggests that the regulatory proteins potentiate the effects of A1-type light chains.

If A1-type ELCs confer a higher affinity for F-actin on S1s carrying them, then there are two potential mechanisms for this effect. Either the ELC could, in some way, affect the actin binding sites in the heavy chain and increase their affinity (since the ELC is far from these sites (Rayment *et al.* 1993b) any such effect would have to be transmitted through the molecule) or the ELC itself could provide an additional contact with actin - presumably in the 40-odd residues unique to A1.

1.4.5.2 Evidence for direct binding of A1-type ELCs to actin.

Sutoh (Sutoh, 1982) used chemical cross-linking to probe the interaction between S1 and F-actin with the zero-length cross-linker EDC, and fluorescently labelled proteins. Among other results he found a 67 kDa product which he assigned as an A1-actin product. The product contained actin (shown by using fluorescent actin), but this study did not prove the involvement of A1 nor did it compare S1(A1) with S1(A2).

The work was extended (Labbé *et al.* 1986) using the more hydrophobic zero-length cross-linker EEDQ. This study showed that the 67 kDa product does indeed result from an A1-actin product: if either actin or A1 is labelled the label is seen in this product. The product is seen only with S1(A1) and not with S1(A2). These workers attempted to cross-link the free light chain to F-actin using EDC and did not succeed (although it would cross-link to actin when in complex with the 20 kDa heavy chain fragment). However, it should be noted that a rather complex cross-linking procedure was used in which one protein was reacted with EDC and then added to the other. The procedure is claimed to give more control over the reaction (as only one protein is exposed to the cross-linker) but it suffers from the drawback that if two species are slow coming to equilibrium, then they might not do so before the relatively rapid decay of the cross-linker-protein derivative has occurred.

The cross-linker EEDQ is not required for the formation of the 67 kDa product - EDC works equally well (Andreev and Borejdo, 1995). These workers confirmed the assignment of this product using fluorescently labelled proteins and showed that the amount of product depends on the degree of saturation of actin with S1(A1): as the S1:actin ratio approaches 1:1 the amount of product drops almost to zero (under physiological conditions the ratio is much less than 1:1). The simplest explanation for this is that the contact made by the ELC to actin is with a different actin monomer to the one the heavy chain contacts. The heavy chain-actin interaction is of higher affinity than the ELC-actin interaction and, therefore, at high degrees of saturation effectively competes with the ELC-actin interaction and is the only contact observed. Both can form at lower degrees of saturation (figure 1.13).

1D-NMR studies on the S1 isoenzymes (Trayer *et al.* 1987) and free A1-type ELCs (Henry *et al.* 1985b; Henry *et al.* 1985a) in the absence of actin, show relatively sharp resonances which can be tentatively assigned to (mainly) lysine, alanine and trimethylalanine residues. These residues occur in the N-terminus and similar spectra are obtained with peptides from this region (Henry *et al.* 1985b; Bhandari *et al.* 1986; Trayer *et al.* 1987, Drs HR Trayer and KJ Smith, unpublished data) and are not seen in S1(A2) or free A2. This suggests that this region in A1 is relatively mobile in solution. It also provides a means of monitoring any direct interaction with actin. If the light chain binds actin then the mobility of this segment will be reduced and the close proximity of another protein will provide additional relaxation pathways. Both effects will result in the broadening of resonances from affected residues. This is precisely what is seen in S1(A1) (Trayer *et al.* 1987), in free A1 (Henry *et al.* 1985b), in an N-terminal thrombic fragment of A1 (Henry *et al.* 1985b), in peptides corresponding to the first 32 residues of A1 (Trayer *et al.* 1987) and in shorter peptides (1-10 and 1-6) (Drs HR Trayer and KJ Smith, unpublished data).

NMR studies can also provide a clue about the site of interaction on actin. Paramagnetic spin labels (ie compounds with unpaired electrons) result in dramatic broadening of NMR signals resulting from nuclei which are close (< 1.5 nm) in space.

Using actin labelled at Cys 374 (near the C-terminus) with a spin label, it was shown (Trayer *et al.* 1987) that this probe affected the N-terminal residues of A1 in S1(A1) and in the 1-32 peptide. This suggests that the C-terminus of actin is close to the site of interaction with the N-terminus of A1.

The most direct visualisation of the ELC-actin interaction comes from electron-microscopy image reconstructions. Milligan and co-workers (Milligan *et al.* 1990) compared image reconstructions of acto-S1 using S1(A1) and S1(A2). They showed that, when using S1(A1) additional electron density is found close to the surface of actin that was not seen in S1(A2) (figure 1.14). This density, while associated with S1 was not actually connected to the bulk of S1, suggesting a concentration of mass close to the actin surface connected by a relatively light segment to the rest of the light chain. It should be pointed out that this change in mass could result, in part, from conformational changes in actin at the binding site. These results have been confirmed recently (Waller *et al.* 1995).

Labelling actin at Cys374 with an (electron dense) undecagold derivative shows that the interaction site is close to the site of labelling. That the effect can be seen at 1:1 S1:actin ratios is in conflict with the cross-linking data (Andreev and Borejdo, 1995) which suggests that little A1-actin interaction occurs at this ratio. This conflict needs to be resolved.

1.4.6 Rôle of the Proline-Rich Segment.

Data from NMR experiments suggests that the first 10 residues of A1 are involved in the interaction with actin (see section 1.4.2). This poses the question of what the function of the remaining 30 residues of the N-terminal extension is.

The sequence of these 30 residues is dominated by a series of (Xxx-Pro) (where Xxx is usually Ala) repeats. NMR studies on this system (Bhandari *et al.* 1986) which measured relaxation times of resonances assigned to this segment and one-dimensional nOes showed that it adopts an extended, rod-like structure of limited flexibility with the

proline residues in the trans conformation. A similar result was obtained with the bacterial periplasmic protein TonB which also has an (Xxx-Pro) rich sequence (Brewer *et al.* 1990).

Further evidence of the ability of this sequence to form extended, inflexible structures comes from X-ray crystallographic analysis of the model peptide $(Aib\text{-}Pro)_4$ (where Aib is α-aminoisobutyric acid or $C^{\alpha,\alpha}$-dimethylglycine) (Di Blasio *et al.* 1992) and from molecular modelling of the (Ala-Pro) rich segment from rabbit skeletal A1 (Abillon *et al.* 1990). By adopting such a structure this "spacer arm" can position the actin binding site on the surface of actin and bridge the gap between the ELC and actin which is apparent from the models of acto-S1 derived from the crystal structures (Waller *et al.* 1995).

A rod-like structure can also confer thermodynamic advantages. The affinity of any molecular interaction depends on the free energy (ΔG) released (or absorbed) on binding. This in turn depends on the enthalpy of interaction (ΔH) and on the entropy (ΔS) - which reflects the degree of order in the system (which includes solvent molecules which may be bound or displaced during the process of interaction). The three parameters are related:

$$\Delta G = \Delta H - T.\Delta S$$

where T is the absolute temperature. Reactions are energetically favourable when ΔG is negative. Clearly this will be favoured if ΔH is negative (ie energy is released on binding) and ΔS is positive (ie the system becomes more disordered). When two macromolecules interact, ΔH is generally negative (since new contacts are made between the molecules) and the situation is thus controlled by ΔS. The formation of the complex results in a more ordered system (reducing the entropy), but can result in the release of tightly bound solvent molecules (increasing the entropy). In the case of the light chain, the interaction is not believed to be particularly strong (ie ΔH is not highly negative), but the fact that the (Xxx-Pro) region is already very highly ordered means that much of the entropic cost of complex formation has already been paid (Williamson, 1994). The spacer-arm thus effectively increases the affinity of A1 (in S1(A1)) for actin

(but not the free light chain, since the flexibility of the arm only needs to be constrained if it has to span the gap between S1 and actin).

There is an alternative (but equivalent), kinetic way of looking at this situation. If the actin binding site were on a flexible arm then its search for its target would be in three dimensions. Placing it on a rigid stick reduces this search to one in two dimensions. Again this argument applies only to A1 in S1(A1); A1 in solution will always have to search through three dimensions.

1.4.7 Correlation of Actin Binding by A1 to Kinetic Modulation.

Given that A1-type ELCs confer kinetic properties on S1 which are different to those conferred by A2, and that A1 may bind directly to actin and A2 does not, it is tempting to suggest that actin binding by A1 results directly in kinetic modulation. In order to establish this correlation, it would be necessary to demonstrate that loss of the actin binding function of the molecule also results in loss of kinetic modulation. Several investigators have attempted to do this by using peptides coding for the postulated actin binding site and proteolytically cleaved A1-type ELCs.

A peptide corresponding to residues 5 to 14 of human ventricular ELC (an A1-type ELC) is reported to result in a 40% increase in force at activating calcium concentrations when incubated with skinned muscle fibres (Morano *et al.* 1995). The peptide concentrations used in this study were of the order of 10^{-12} M. No evidence was given that the peptide penetrates the fibres and few controls were reported. It seems unlikely that such a low concentration of peptide could have such a dramatic effect (especially considering that the peptide concentration within the fibre may be even lower). This concentration will be orders of magnitude lower than the effective concentration of ELC within the thick filament (in the order of 10^{-4} M (Yates and Greaser, 1983)) and yet these authors report a maximum effect at these levels of peptide.

Another group treated S1(A1) with papain which cuts A1 at position 13 to give a fragment referred to by the authors as A1′. S1(A1′) has actin activated MgATPase kinetics similar to S1(A2) (Hayashibara and Miyanishi, 1994). They were also able to purify the 13 residue fragment from the N-terminus of A1 (N-Pep) and use this in competition experiments with S1(A1). Their treatment is based on a limited number of points, but appears to suggest that, in the presence of N-Pep, S1(A1) gives MgATPase kinetics which are more S1(A2)-like. This effect is dependent on the concentration of N-Pep. However, the peptide concentration data is treated as a simple case of competitive inhibition which it clearly is not since the main criterion for competitive inhibition (that k_{cat} is not dependent on [inhibitor] (Cornish-Bowden, 1979)) is not met. Clearly this approach shows promise, but requires a more rigorous treatment.

1.5 AIMS.

Overall Aim:

To better understand the rôle of the ELC in modulating the motor activity of myosin.

Specific Aims:

1. To determine if an actin binding site at the N-terminus of A1-type essential light chains is responsible for kinetic modulation.

2. To locate this site more precisely.

3. To investigate the rôle of the proline rich region. Does it function as a spacer arm responsible for correctly positioning the actin binding site?

4. To investigate the rôle of individual amino acid residues within the actin binding site.

5. To quantify the actin-A1 interaction.

6. To investigate the rôle of the regulatory light chain from *Drosophila* flight muscle. Does it function like a vertebrate striated muscle A1-type ELC.

1.6 STRATEGY

The approach taken was a protein engineering one. The human cardiac myosin essential light chain (an A1-type ELC) was over-expressed in the bacterium *E. coli* and a purification strategy developed. The recombinant light chain was then introduced into rabbit skeletal myosin subfragment 1 to produce hybrid S1. An assay system which clearly differentiates between S1s carrying A1- or A2-type ELCs was developed along with a chemical cross-linking method which enabled the binding of the ELC to actin (both as "free" light chain and in complex to the heavy chain) to be monitored.

The region responsible for kinetic modulation was located by making a series of N-terminal deletion mutants, hybridising these mutants into S1 and assaying the actin activated MgATPase activity of the hybrids in order to assess which kinetic class (ie A1- or A2-like) the light chain belongs to. The actin binding site was located by attempting to cross-link these deletion mutants to actin. The hypothesis that the proline-rich region functions as a spacer arm was tested by varying its length and the rôle of individual amino acids in the actin binding site was assessed by site directed mutagenesis. An attempt to quantify the ELC-actin interaction was made using a surface plasmon resonance biosensor.

Protein engineering proved to be a powerful method to address this important problem and the results from this study clarify many of the questions posed in section 1.5 and open the door to further investigations (see General Discussion, Chapter 7).

Chapter Two:

Materials & Methods

2A. MATERIALS

2A.1 MOLECULAR BIOLOGY REAGENTS.

DNA modifying enzymes (restriction endonucleases, T4 DNA ligase); Calf alkaline phosphatase	New England Biolabs
Taq DNA polymerase	Boehringer Mannheim
pET vectors; bacterial strains	AMS Biotechnology
[α-^{35}S]dATP; Sequenase™ Quick-Denature™ Plasmid Sequencing Kit	Amersham International
Sequencing gel mix and buffers	National diagnostics
Growth media etc	Difco
Phenol/Chloroform	CamLab
Wizard™ MiniPrep Kit	Promega
Agarose (genetic technology grade)	ICN Biochemicals

2A.2 PROTEIN BIOCHEMISTRY MATERIALS

DEAE Sepharose FastFlow	Pharmacia Biotech
SP Trisacryl	IBF Biotechnics
1,5-IAEDANS, MPB	Molecular Probes
NHS biotinylated actin	Cytoskeleton
PVDF membrane	Millipore
Protein molecular mass markers	NEB
Ammonium sulphate (low in heavy metal for enzyme work); Ammonium chloride	BDH Chemical Limited
Bovine pancreatic DNase-I; EDC (>99% purity)	Fluka
α-Chymotrypsin (TLCK treated)	Worthington Chemical Corporation
Aquacide	Calbiochem

2A.3 SPR MATERIALS

CM dextran sensor chips, SA5 sensor chips (with pre-immobilised streptavidin), a GST-fusion capture kit (which includes a high specificity, high affinity monoclonal antibody to GST) and the reagents for amine and thiol immobilisation were obtained from Pharmacia Biosensor.

2A.4 OTHER REAGENTS

All other reagents were purchased from reputable suppliers and unless indicated below were used without subsequent treatment.

2B. MOLECULAR BIOLOGY METHODS

Molecular biology methods were based on established, published techniques (Sambrook *et al.* 1989) and the reader is referred to these sources for a full description. A full description of the contents of all buffers and media are given in section 2B.9.

All manipulations involving DNA solutions, growth media etc were performed using sterile media and buffers. Sterilisation was achieved by autoclaving at 15 $lb.in^{-2}$ for 15 minutes.

2B.1 PREPARATION & PURIFICATION OF OLIGODEOXYNUCLEOTIDES.

2B.1.1 Synthesis of Oligodeoxynucleotides.

Oligonucleotides were synthesised by Alta Bioscience (School of Biochemistry, The University of Birmingham. Director Dr John Fox) using solid phase methods (Gait, 1984) on machines designed "in house". They were supplied as dried material and stored at -20°C until required.

2B.1.2 Purification of Oligodeoxynucleotides.

Oligonucleotides were purified by butanol extraction. Briefly, the dried oligo as supplied by Alta Bioscience was taken up in 1 ml sterile water. 0.5 ml was removed and frozen and an equal volume of water saturated butanol was added to the remainder. The mixture was vortexed thoroughly for at least 30 seconds and then centrifuged for 5 minutes at top speed in a bench-top centrifuge. The lower, aqueous layer was removed, retained and subjected to two further rounds of extraction.

The purified oligo was then concentrated by ethanol precipitation and taken up in 25 µl sterile water. The concentration was then estimated by measuring the absorbence at 260 nm of a 1/1000 dilution of the stock. An A_{260} value of 1.0 corresponds to an oligo concentration of approximately 20 $\mu g.ml^{-1}$ and this value was used exclusively for

estimating the concentration. Stock oligo solutions (typically 3 to 10 $mg.ml^{-1}$) were then diluted with sterile water to give solutions of around 1 $mg.ml^{-1}$ (0.84 $mg.ml^{-1}$ for PCR reactions - see section 2B.2).

2B.1.3 Sequences of Oligodeoxynucleotides.

There follows a list of oligos used during the course of this investigation and a brief description of what they were used for.

D2218: 5' Human Atrial ELC (Wild Type)

GGG AAT TCA TAT GGC TCC CAA GAA GCC
1 4 7 10 13 16 19 22 25

Bases 8 to 13 encode an NdeI recognition site; 11 to 27 correspond to the gene sequence (Arnold *et al.* 1988) for human atrial ELC with 11 to 13 being the start codon. This oligo was used for PCR amplification (section 2B.2) of the wild type gene with the introduction of an NdeI restriction enzyme site at the start of the gene to facilitate in-frame cloning into the expression vector.

D2219: 3' Human Atrial ELC (Wild Type)

AAG GAT CCA GAC TCT GCT TCA CCC TG
1 4 7 10 13 16 19 22 25

Bases 3 to 8 encode a BamHI site; 11 to 26 correspond directly to the human atrial ELC sequence with 11 to 13 being the stop codon. This oligo was used for PCR amplification of the wild type (and N-terminal mutants) DNA sequence and to introduce a BamHI site 3' to the gene.

D3017: 5' Human Atrial ELC (A1-11 Mutant)

CCG AAT TCA TAT GGC CAA GCC AGC TC
1 4 7 10 13 16 19 22 25

This oligo was used to amplify a fragment of the wild type human atrial ELC sequence whose protein coding sequence begins at amino acid 11. Bases 8 to 18 are an NdeI restriction site.

D3016: 5' Human Atrial ELC (A2-like Mutant)

CCG AAT TCA TAT GGA CTT CAC TGC CGA C
1 4 7 10 13 16 19 22 25 28

This oligo was used to amplify a fragment of the wild type human atrial ELC sequence whose protein coding sequence begins at amino acid 44. Bases 8 to 18 are an NdeI restriction site.

D5880: 5' Human Atrial ELC (A1.ΔXP Mutant)

AAA CAT ATG GCT CCC AAG AAG CCT GAG CCT AAG AAG GAG GCA
1 4 7 10 13 16 19 22 25 28 31 34 37 40
GAC TTC ACT GCC G
43 46 49 52 55

This oligo was used to amplify a segment of DNA which corresponded to the wild type sequence but lacking bases 37 to 138. Bases 7 to 42 of the oligo correspond to bases 1 to 36 of the wild type sequence and bases 43 to 55 correspond to bases 139 to 151 of the wild type gene. Bases 4 to 9 encode an NdeI site. Full details of the use of these oligo are given in Paper II.

D6126: Human Atrial ELC (A1.2XP Mutant) - "Upper" Strand Insert

TGA GCC AGC TCC AGC TCC AGC TCC AGC TCC TGC ACC AGC
1 4 7 10 13 16 19 22 25 28 31 34 37
CCC TGC CCC AGC TCC
40 43 46 49 52

Bases 3 to 54 of the oligo correspond to bases 51 to 92 of the wild type human atrial ELC sequence. Full details of how this oligo and its partner (D6127) were used are given in Paper II.

D6127: Human Atrial ELC (A1.2XP Mutant) - "Lower" Strand Insert

TCA GGA GCT GGG GCA GGG GCT GGT GCA GGG GCT GGA GCT GGA
1 4 7 10 13 16 19 22 25 28 31 34 37 40
GCT GGA GCT GGC
43 46 49 52

Bases 1 to 54 of the oligo sequence correspond to bases 52 to 95 of the wild type gene sequence.

D6751: 5' Human Atrial ELC (A1V Mutant)

GGG TCA TAT GGT TCC CAA GAA GCC TGA
1 4 7 10 13 16 19 22 25

Used for PCR amplification of human atrial ELC carrying the mutation A1V (see Paper III). Bases 5 to 10 encode an NdeI site and bases 11 to 13 introduce the change [GCT(Ala)→GTT(Val)].

D6752: 5' Human Atrial ELC (+D0 Mutant)

AAT TCA TAT GGA CGC TCC CAA GAA GCC TGA
1 4 7 10 13 16 19 22 25 28

Used for PCR amplification of human atrial ELC carrying the mutation +D0 (see section 5.2B.2.1). Bases 5 to 10 encode an NdeI site and bases 11 to 13 introduce the change [GAC(Asp) is introduced into the sequence].

D8360: 5' Human Atrial ELC (K4R Mutant)

AAT TCA TAT GGC TCC CAA GCG CCC TGA GCC TAA
1 4 7 10 13 16 19 22 25 28 31

Used for PCR amplification of human atrial ELC carrying the mutation K4R (see Paper III). Bases 5 to 10 encode an NdeI site and bases 20 to 22 introduce the change [AAG(Lys)→CGC(Arg)].

D8361: 5' Human Atrial ELC (K4E Mutant)

AAT TCA TAT GGC TCC CAA GGA GCC TGA GCC TAA
1 4 7 10 13 16 19 22 25 28 31

Used for PCR amplification of human atrial ELC carrying the mutation K4E (see section 5.2B.2.2). Bases 5 to 10 encode an NdeI site and bases 20 to 22 introduce the change [AAG(Lys)→GAG(Glu)].

D8362: 5' Human Atrial ELC (K4A Mutant)

AAT TCA TAT GGC TCC CAA GGC GCC TGA GCC TAA
1 4 7 10 13 16 19 22 25 28 31

Used for PCR amplification of human atrial ELC carrying the mutation K4A (see Paper III). Bases 5 to 10 encode an NdeI site and bases 20 to 22 introduce the change [AAG(Lys)→GCG(Ala)].

D9397: 5' Human Atrial ELC (K4D Mutant)

AAT TCA TAT GGC TCC CAA GGA CCC TGA GCC TAA
1 4 7 10 13 16 19 22 25 28 31

Used for PCR amplification of human atrial ELC carrying the mutation K4D (see Paper III). Bases 5 to 10 encode an NdeI site and bases 20 to 22 introduce the change [AAG(Lys)→GAC(Asp)].

D9398: 5' Human Atrial ELC (K3A Mutant)

AAT TCA TAT GGC TCC CGC GAA GCC TGA GCC TAA
1 4 7 10 13 16 19 22 25 28 31

Used for PCR amplification of human atrial ELC carrying the mutation K3A (see Paper III). Bases 5 to 10 encode an NdeI site and bases 17 to 19 introduce the change [AAG(Lys)→GCG(Ala)].

2B.2 POLYMERASE CHAIN REACTION.

The polymerase chain reaction (Saiki *et al.* 1988) was used to amplify DNA fragments prior to sub-cloning into expression vectors, to introduce restriction enzyme sites 5' and 3' to these fragments and to introduce mutations into the first 15 bases of the gene. In all cases the thermostable DNA polymerase used was Taq Polymerase. The principal of the technique is illustrated in Figure 2.1a.

For most of the reactions the oligos used contained sequence that overlapped with the fragment to be amplified and overhangs encoding restriction enzyme sites. A two stage

amplification process was used (figure 2.1b). The annealing temperature for the whole oligo ($T_{m,long}$) and for the overlapping sequence ($T_{m,short}$) was calculated using the equation:

$$T_m = 64.9 + 0.41(\%G+C) - 600/n \text{ (Sambrook } et\ al. \text{ 1989)}$$

Where: (%G+C) is the percentage of G and C nucleotides in the oligo and n is the number of bases.

For the first three cycles $T_{m,short}$ was used and for the remaining twenty seven $T_{m,long}$ was used. A typical PCR reaction (used to amplify the wild type human atrial ELC) is shown below.

The following reaction mix was made up:

Sterile water	165 µl
Taq Buffer (×10)	20 µl
dNTP mix (10 mM)	4 µl
5'-Primer (0.84 $mg.ml^{-1}$)	3 µl
3'-Primer (0.84 $mg.ml^{-1}$)	3 µl
Taq DNA Polymerase	1 µl

The mix was then split between four separate tubes to which 0, 0.01, 0.1 and 1 ng template DNA were added. Approximately 50 µl of mineral oil was then added to prevent evaporation during themal cycling. The following PCR programme was used.

No. of Cycles	Temp. 1 / °C	Time 1 / s	Temp. 2 / °C	Time 2 / s	Temp. 3 / °C	Time 3 / s
3	94	30	55 ($T_{m,short}$)	60	72	60
27	94	30	60 ($T_{m,long}$)	60	72	60
1	72	420				
1	4	∞				

2B.3 General DNA Manipulation Techniques.

2B.3.1 Phenol Chloroform Extraction.

Phenol-Chloroform extraction was used to remove contaminating proteins (including restriction enzymes) from DNA solutions.

An equal volume of phenol-chloroform was added, the mixture vortexed thoroughly and then centrifuged for 5 minutes at top speed in a bench-top centrifuge. The upper (aqueous) layer was then carefully removed and retained.

2B.3.2 Ethanol Precipitation

Ethanol precipitation was used to recover DNA from relatively large volumes of solution following purification etc.

1/10th volume 3 M sodium acetate was added, followed by 2 to 3 volumes of absolute ethanol and the tube inverted several times to mix the contents. The mixture was then cooled to -70°C for at least 10 minutes and then centrifuged at 4°C at 15000g for 10 minutes. The supernatant was gently poured away and the remaining liquid allowed to drain away. The pellet was washed in cold (-20°C) 70 %(v/v) ethanol and centrifuged immediately at 4°C for 10 minutes at 15000 g. Again the pellet was retained and dried by leaving the tube on a 42°C heating block with the lid open until dry. (This method was found to be preferable to using a GyroVac, which often took longer and carried the associated risk of irretrievable loss of the pellet.)

2B.3.3 Restriction Enzyme Digests.

Restriction digests were typically carried out using around 2 to 4 units of enzyme (manufacturer's unit definition) per μg DNA in the buffer recommended and supplied by the manufacturer. Reactions were incubated at 37°C for about one hour.

2B.3.4 Ligation of Cohesive Ended DNA.

Ligations were carried out using 100 units of T4 DNA Ligase (manufacturer's unit definition) in the buffer recommended and supplied by the manufacturer. When attempting to ligate fragments into expression vectors it was found that the reaction is sensitive to both the quality of the cut vector (see section 2B.5.3) and the ratio of insert to vector. Ligations were left for at least one hour (and usually overnight) at room temperature.

2B.3.5 Agarose Gel Electrophoresis.

DNA fragments were analysed by gel electrophoresis in 1 to 2% agarose. This technique separates DNA molecules on the basis of size and the size of fragments was estimated by comparison to commercially obtained molecular mass markers. Gels were run at 20 $V.cm^{-1}$ in TAE buffer (section 2B.9) and the agarose was also made up in this buffer. Fragments were visualised by staining in 25 $\mu g.ml^{-1}$ ethidium bromide solution (typically for about 10 minutes) and then viewing under UV light.

2B.4 Bacterial Cell Lines & Manipulation.

2B.4.1 Bacterial Cell Lines

Two *E. coli* strains were used: JM101 and BL21(DE3).

JM101 is a "general purpose" strain which permits the growth of transformed plasmids to high copy number. It was used when sub-cloning DNA fragments into the expression vectors and for the preparation of stocks of vectors containing inserts.

BL21(DE3) was used for over-expression of sub-cloned genes. The bacteriophage λ DE3 is integrated into the bacterial chromosome and this lysogen contains the T7 RNA

polymerase gene under the control of the LacUVR5 promotor (Studier *et al.* 1990; Studier and Moffat, 1986)

2B.4.2 Preparation of Calcium Competent Cells.

In order to prepare cells that can be (relatively) efficiently transformed (section 2B.4.3), it is necessary to make them competent. The method used was exclusively that of using calcium chloride (Sambrook *et al.* 1989). Most of the competent cells used in this study were prepared by Miss Sue Brewer.

Bacterial strains were maintained on minimal media plates (no added antibiotics) and single colonies picked and grown overnight, shaking at 37°C in LB medium (section 2B.9) with no added antibiotics. 0.5 ml of this overnight culture was used to inoculate 50 ml LB and this culture was grown for about 2 hours (shaking at 37°C) until A_{600} was about 0.5. The cells were harvested by centrifugation at 5000g at 4°C and resuspended in 20 ml cold 50 mM $CaCl_2$. The cells were left on ice for at least 20 minutes and then harvested as described above. They were the resuspended in 5 ml 100 mM $CaCl_2$, 15 %(v/v) glycerol and stored frozen at -70°C in 500 µl aliquots. Once thawed for use, the cells were not refrozen.

2B.4.3 Transformation of Competent Cells.

The same procedure was adopted for all competent cell lines.

DNA solutions were cooled on ice, prior to the addition of 100 µl of cell suspension. (The cell suspension was thawed slowly on ice.) The mixture was then left on ice for between 30 and 60 minutes, before heat shocking for 2 minutes at 42°C. 1 ml of Luria Betani (LB) medium (section 2B.9) was then added and the cells incubated at 37°C for between 45 and 90 minutes. After this time the cells were pelleted by centrifugation for 1 minute at top speed in a bench top centrifuge and most of the supernatant poured away (leaving around 200 µl). The cells were resuspended in the remaining media and then

plated out on appropriate media (usually MacConkey Agar/Maltose/Ampicillin - section 2B.9). Plates were incubated, upside down, overnight at 37°C.

2B.5 PLASMIDS & PLASMID PREPARATIONS.

2B.5.1 Plasmids

All plasmid vectors used for the expression of recombinant proteins in *E. coli* were pET vectors. These vectors enable overexpression of foreign proteins under the control of a T7 promoter (Studier *et al.* 1990; Studier and Moffat, 1986).

This technology exploits the natural ability of T7 bacteriophage to subvert *E. coli* by using part of the host cell's protein synthesis machinery to its own ends. The bacteriophage expresses a highly efficient RNA polymerase which recognises a specific promoter sequence which is different to any *E. coli* promotor. This enables the 'phage to produce large quantities of mRNA coding for 'phage proteins which are then translated by the host's machinery. The pET vector system exploits this by placing the protein of interest under the control of a T7 promoter. In the expression strain, (BL21(DE3)) a chromosomally encoded copy of T7 RNA polymerase is under the negative control of LacI. When repression is relieved by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (an analogue of the inducer, 1,6-allolactose) the T7 RNA polymerase gene is transcribed and translated. T7 RNA polymerase then transcribes the gene of interest in the plasmid.

pET 11c, the main vector used in this study includes a number of enhancements designed to reduce background expression of potentially toxic proteins prior to induction. There is a copy of the gene for LacI which increases the number of lac repressor molecules in the cell and a Lac repressor binding site, partially overlapping the T7 promotor. This has the effect of tightening control over gene expression. A low resolution map of pET 11c is shown in Figure 2.2.

2B.5.2 Plasmid Preparation from Bacterial Cultures.

Plasmids were prepared from cultures grown in LB medium, at 37°C, shaking overnight by one of two methods.

2B.5.2.1 Alkaline Lysis MiniPrep

This method was the most commonly used method of isolating plasmid DNA.

Cells from 1 to 3 ml of overnight culture were pelleted by centrifugation at top speed in a bench-top centrifuge. The cells were resuspended in 500 µl STE buffer (section 2B.9) and then pelleted again. Following this they were resuspended in 100 µl GET buffer and the cells lysed by adding 200 µl of a freshly prepared mixture of 0.2 M sodium hydroxide and 2% (w/v) SDS solution and left for five minutes. 150 µl of cold potassium acetate solution was added to precipitate the SDS and chromosomal DNA. The mixture was left on ice for ten minutes and the precipitate removed by spinning for 10 minutes at 15000g at 4°C. The supernatant was subjected to phenol-chloroform extraction (section 2B.3.1) and ethanol precipitation (as section 2B.3.2 except that no sodium acetate was added during the first precipitation step). Plasmids were resuspended in 25 µl TE buffer.

2B.5.2.2 Wizard™ MiniPrep

This method gives a much purer product than the alkaline lysis method (which gives DNA contaminated with large quantities of RNA) and was used when quality was crucial - eg for the preparation of cut vector and for DNA sequencing. The kit includes pre-made solutions for cell lysis etc and pre-made columns for the isolation of plasmid DNA. It was used according to the instructions provided by the manufacturer.

2B.5.3 Preparation of NdeI/BamHI cut vector.

Plasmid DNA (25 µl) purified using the Wizard MiniPrep was first digested using NdeI (section 2B.3.3) and then purified by phenol-chloroform extraction (section 2B.3.1) and ethanol precipitation (section 2B.3.2). This allows the second digestion (with BamHI) to also be carried out in its recommended buffer. Following this digestion 5 µl of calf alkaline phosphatase was added to dephosphorylate the cohesive ends produced by the digestions. The mixture was incubated for one hour at 37°C and the resulting cut and CAPped plasmid purified by phenol-chloroform extraction and ethanol precipitation. The cut vector was taken up in 20 µl TE buffer and stored frozen at -20°C until required.

2B.6 DNA SEQUENCING

Two methods were used for double stranded DNA sequencing, both of which were based on the dideoxynucleotide chain-terminating method of Sanger (Sanger *et al.* 1977).

2B.6.1 Automated DNA Sequencing

Automated DNA sequencing was carried out by Alta Bioscience using an Applied Biosystems 373A automatic sequencer and fragments were detected by fluorescent dyes coupled to the terminators. Taq polymerase was used for the extension reactions. This method gave variable qualities of data; this being mainly dependant on the quality of the plasmid DNA.

2B.6.2 Manual DNA Sequencing

This was carried out using Amersham's Sequenase™ Quick-Denature™ Plasmid Sequencing Kit. The kit incorporates pre-made solutions for all stages of the sequencing reaction, including the denaturation of double stranded DNA. It was used exactly according to the manufacturer's instructions.

2B.7 SUB-CLONING INTO EXPRESSION VECTORS.

In order to express the wild type and mutant human atrial ELCs in *E. coli*, it was necessary to introduce appropriate DNA sequences into the expression vectors.

The DNA sequence was amplified using the polymerase chain reaction (section 2B.2) using primers which introduced an NdeI restriction site 5' to the gene and a BamHI site 3' to it. The presence and size of the product was checked by 1% agarose gel electrophoresis (section 2B.3.5) and purified by phenol chloroform extraction (section 2B.3.1) and ethanol precipitation (section 2B.3.2). The product was dissolved in 31 µl of water and subjected to a double restriction enzyme digest (section 2B.3.3) using the enzymes NdeI/BamHI (7 µl of each) in BamHI buffer (5 µl). The digested product was purified by phenol-chloroform extraction and ethanol precipitation and taken up in 20 µl sterile water. The presence and size of the product was again verified by 1% agarose gel electrophoresis. The product was ligated (section 2B.3.4) into NdeI/BamHI cut pET-11c (sections 2B.5.1 and 2B.5.3).

The ligation reaction was used without further purification to transform competent JM101 cells (section 2B.4.3). Plasmid DNA was isolated from all colonies using the alkaline lysis miniprep (section 2B.5.2.1) and screened for the presence of inserts by restriction enzyme digestion with PstI. All the inserts contain a PstI site and the vector contains a naturally occurring site. The presence of the insert is detected by the appearance of an additional band upon 1% agarose gel electrophoresis (figure 2.3). This ligation and screening step is critical and depends on the concentration ratio of insert to cut vector and on the volume of the ligation mix used in the transformation. It was often necessary to vary these two parameters in order to get clones which screened positive.

Positive clones were back-transformed into JM101 cells and stocks of pure plasmid prepared using the Wizard MiniPrep (section 2B.5.2.2) for use in DNA sequencing reactions.

2B.7 EXPRESSION OF HETEROLOGOUS PROTEINS IN *E. COLI*

2B.7.1 Small-Scale "Test" Expression.

Plasmids known to contain inserts (typically 0.5 μl) were used to transform competent BL21(DE3) cells (section 2B.4). Single colonies were picked and grown overnight, shaking at 37°C in 5 ml LB media supplemented with 100 $\mu g.ml^{-1}$ ampicillin. 100 μl of these cultures was sub-cultured into 5 ml NZCYM medium (section 2B.9) supplemented with 100 $\mu g.ml^{-1}$ ampicillin and grown for a further 2 to 3 hours. After this time the cells were induced by the addition of IPTG to a final concentration of 0.4 mM and left to grow for 2 to 5 hours. At all stages the cell density was monitored by measuring the A_{600} value; cells were not induced until this value exceeded 0.6. 1 ml aliquots of cells were spun down in a bench-top centrifuge and resuspended in gel loading buffer (section 2B.9). The success (or otherwise) of protein expression was judged by comparing SDS-PAGE (section 2C.2.1) profiles of cells extracts before and after induction. Successful induction was indicated by the appearance of an additional band of approximately correct molecular mass.

2B.7.2 Large-Scale Expression for the Preparation of Recombinant Protein.

BL21(DE3) colonies (previously transformed with the appropriate plasmid) were picked and grown overnight in 40 ml of LB media supplemented with 100 $\mu g.ml^{-1}$ ampicillin shaking at 37°C. These cultures were diluted into 500 ml NZCYM (pre-warmed to 37°C) supplemented with 100 $\mu g.ml^{-1}$ ampicillin and shaken until A_{600} exceeded 1.0 (typically 2 to 3 hours). After this time the cells were induced by the addition of IPTG to a final concentration of 0.4 mM and left to grow for 2 to 5 hours. Protein expression was checked by SDS-PAGE analysis as described in section 2B.7.1.

The cells were then harvested by centrifugation at 8000g, resuspended in 25 mM TEA.HCl, pH 7.5 and re-pelleted by centrifugation at 8000g. The supernatant was discarded and the cells frozen at -70°C and stored at -20°C until required.

2B.9 Growth Media, Buffers & Other Solutions.

2B.9.1 Growth Media etc.

Luria-Bertani (LB) Medium

Luria-Bertani Medium	25 g

This was made up to 1 L and sterilised by autoclaving.

NZCYM Medium

NZ Bacto Tryptone	10 g
Sodium Chloride	5 g
Bacto Yeast Extract	5 g
Casamino Acids	1 g
Magnesium Chloride (Hydrated Salt)	2 g

The mix was made up to 1 L and sterilised by autoclaving.

MacConkey Agar/Maltose/Ampicillin Agar Plates

MacConkey Agar Base	20 g
Maltose	5 g

This was made up to 500 ml and sterilised by autoclaving, allowed to cool to about 60°C before addition of solid ampicillin (0.05 g) and poured into petri dishes to a depth of about 4 mm. This volume made about 20 plates.

2B.9.2 Buffers.

All buffers (except the agarose gel running buffer) were sterilised by autoclaving.

TE Buffer

Tris	10 mM
Sodium EDTA	1 mM

The pH was adjusted to 8.0 with concentrated HCl.

STE Buffer

Sodium Chloride	0.1 M
Tris	10 mM
Sodium EDTA	1 mM

The pH was adjusted to 8.0 with concentrated HCl.

GET Buffer

Glucose	50 mM
Tris	25 mM
Sodium EDTA	10 mM

The pH was adjusted to 8.0 with concentrated HCl.

Sodium Acetate Solution

Sodium Acetate	3 M

The pH was adjusted to 5.2 with glacial acetic acid.

Potassium Acetate Solution

Potassium Acetate	3 M
Acetic Acid	2 M

TAE Agarose Gel Running Buffer

Tris	40 mM
Acetic Acid	40 mM
Sodium EDTA	1 mM

The pH was adjusted to 8.0 with concentrated HCl.

2B.9.3 Other Solutions.

SDS Gel Loading Buffer

Tris	50 mM
SDS	2 %(w/v)
Bromophenol Blue	0.1 %(w/v)
Glycerol	10 %(v/v)

The pH was adjusted to 6.8 with concentrated HCl.

β-Mercaptoethanol is added to a final concentration of 5 %(v/v) immediately prior to use.

2C PROTEIN BIOCHEMISTRY METHODS

2C.1 PROTEIN MANIPULATIONS.

All protein manipulations were carried out on ice, or in a cold room unless otherwise stated. A full description of all buffers etc can be found in section 2C.9.

2C.1.1 Column Chromatography.

All column chromatography was carried out with commercially available resins. Fractions were assayed by measuring the UV absorbance at 280 nm and by SDS-PAGE (section 2C.3.1).

2C.1.2 Ammonium Sulphate Fractionation.

Solid ammonium sulphate was added slowly with stirring (to avoid the risk of high local concentrations) to the desired concentration (determined from standard tables) and the precipitate collected by centrifugation at 15000g at 4°C for 15 minutes. Since the dissolution of ammonium sulphate results in changes of volume, it is necessary to redetermine the volume after each round of fractionation.

2C.2 PROTEIN PREPARATIONS.

2C.2.1 Protein Preparation from Rabbit Skeletal Muscle

2C.2.1.1 Myosin SubFragment 1.

Myosin was prepared according to the method of Trayer and Perry (Trayer and Perry, 1966) and S1 prepared by chymotryptic digestion (Weeds and Taylor, 1975) and the isoenzymes separated on SP-Tris Acryl™ (Trayer and Trayer, 1988).

Myosin was extracted from the longissimus dorsi muscle of New Zealand White rabbits by a method which exploits the fact that myosin forms filaments (and consequently precipitates) at low ionic strength, but does not (and is soluble) at higher salt concentrations. The muscle was minced and extracted for 20 minutes on ice with a three volume excess of 0.6 M sodium chloride, 50 mM β-mercaptoethanol. The residue was removed by centrifugation at 8000 g for 10 minutes and retained and frozen for the extraction of actin (section 2C.2.1.2). The supernatant was filtered, under low suction, through a glass wool pad previously washed with 0.6 M sodium chloride. The filtrate was stirred into 10 L of cold, distilled water containing 50 mM β-mercaptoethanol. The myosin precipitate was allowed to settle and the supernatant siphoned off before centrifugation at 8000 g for 5 minutes to collect the myosin. The total volume of precipitate was estimated and solid sodium chloride added to a final concentration of 0.6 M and β-mercaptoethanol to a final concentration of 50 mM. This mixture was stirred on ice until the myosin had dissolved and the resulting solution stirred into a 15 to 20-fold excess of cold, distilled water containing 50 mM β-mercaptoethanol.

Once again the precipitate was collected and redissolved as described above. The myosin solution was centrifuged at 80000g for one hour. The supernatant was filtered through a glass wool pad pre-washed with 0.6 M sodium chloride. The product was stored in a sealed bottle in the fridge until required.

Typical yields were 9 g myosin per 200 g muscle.

The myosin solution produced by the method described above was used in the preparation of S1. The solution was dialysed overnight against a 25-fold excess of filament buffer (section 2C.9). The filaments were equilibrated in a conical flask to 25°C on a water bath and then transferred to a magnetic stirrer at room temperature. α-Chymotrypsin was added to a concentration of 50 μg per ml myosin solution and the reaction mix stirred for 8 minutes. The reaction was then stopped by the addition of PMSF to a concentration of 2 mM and stirred for a further 2 minutes. The mixture was then centrifuged for 60 minutes at 80000g to remove the rod fragment (see section 1.1.4.1). The supernatant (containing S1) was dialysed against 1 L of separation buffer

(section 2C.9) containing 0.5 mM PMSF for 2 hours on a rocking dialyser, then 1 L separation buffer containing 0.2 mM PMSF for 2 hours on a rocking dialyser and finally against 5 L separation buffer containing 0.1 mM PMSF overnight. The dialysate was then clarified by centrifugation at 80000g for 2 hours.

Typical yields were 1.5 g crude S1 per 9 g myosin.

The crude S1 was applied to an SP-Tris Acryl column (bed volume 800 ml) equilibrated in the separation buffer. The column was washed at a flow rate of 1 $ml.min^{-1}$ and fractions (of size 5 to 6 ml) collected. When unbound material had washed through (determined by monitoring the value of A_{280}) the bound protein was eluted with a 2 × 600 ml 0 to 0.2 M sodium chloride gradient. Fractions containing pure S1(A1) or pure S1(A2) were identified using SDS-PAGE analysis (section 2C.2.1). These fractions were pooled and concentrated on Aquacide such that the final concentration of each isoenzyme was of the order of 10 $mg.ml^{-1}$. Each pool was then dialysed against a 50-fold volume excess of 25 mM TEA.HCl, pH 7.5, 0.25 mM DTT overnight and centrifuged at 80000g for 90 minutes. The final concentration was determined (section 2C.2.2) and solid sucrose was added to a concentration of 4 mg sucrose per mg S1 and solid DTT to a concentration of 2 mM. The solution was divided into aliquots containing approximately 20 mg S1 and the aliquots were then shell frozen in dry ice/ethanol and stored, frozen at -20°C.

Typical yields were 300 to 400 mg of each isoenzyme per 1.5 g crude S1.

2C.2.1.2 Actin

Rabbit skeletal muscle actin was prepared according to the method of Spudich and Watt (Spudich and Watt, 1971).

Muscle residue (about 200 g) from which the myosin has been previously extracted was extracted for 15 minutes in 3 volumes of 50 mM sodium hydrogencarbonate. The residue was then collected by centrifugation at 8000 g for 20 minutes and then

resuspended in 10 volumes of 1 mM EDTA and stirred well with a glass rod. The residue was again collected by centrifugation as before and extracted twice in 10 volumes of distilled water. It was then extracted twice with 2 volumes of acetone and then with one volume of acetone. The fibrous mass was spread out on filter paper and left overnight to dry. This muscle acetone powder was stored in a stoppered bottle at -20°C until required.

Muscle acetone powder (5 g) was extracted for 30 minutes in 100 ml buffer A (section 2C.9) and the residue re-extracted in 200 ml buffer A in a Waring blender. Solid matter was then removed by centrifugation at 10000 g for 30 minutes. Solid sodium chloride was added to the supernatant to a final concentration of 50 mM along with magnesium chloride to a final concentration of 2 mM. The actin was left to polymerise for 2 hours. After this time, solid sodium chloride was added to a final, total concentration of 0.6 M (this dissociates the majority of the actin binding proteins) and the mixture left for at least one hour before the F-actin was collected by centrifugation at 100000g for 3 hours. The supernatant was discarded and the F-actin pellets taken up in about 20 ml buffer A. The actin was then depolymerised by dialysis against 2 L of buffer A on a rocking dialyser for 48 hours. The resulting solution was clarified by centrifugation at 100000g for 2 hours and then freeze dried.

Typical yields were 200 mg actin per 5 g muscle acetone powder.

2C.2.1.3 A1 and A2 Light Chains.

All rabbit skeletal A1 and A2 light chains used in this study were prepared and kindly donated by Dr HR Trayer. They were prepared by the urea dissociation method (Henry *et al.* 1985a). Briefly, the light chains are dissociated from myosin using 9 M urea, separated from the denatured heavy chain by ethanol precipitation and from each other on DEAE-Sephadex in the presence of 4 M urea. They are then renatured by dialysis to remove the urea, freeze dried from ammonium hydrogencarbonate and stored at -20°C as freeze dried powders.

2C.2.2 Preparation of G-Actin:DNase I.

Freeze dried actin powder (about 10 mg) was reconstituted by dissolving in 1 ml buffer A (section 2C.9) in the presence of 1 mg DTT and then dialysed (on a rocking dialyser) overnight. The dialysate was clarified by centrifugation at 80000g for 30 minutes.

Bovine pancreatic DNaseI was reconstituted by dissolving about 10 mg in 5 mM TEA.HCl, pH 7.5, 0.1 mM calcium chloride, 0.1 mM PMSF, 0.25 mM DTT and dialysing against this buffer overnight. The dialysate was clarified by centrifugation at 15000g for 10 minutes.

G-actin and DNaseI solutions were mixed together such that the actin:DNaseI molar ratio was 1:1.3 and the mixture left at room temperature for one hour before use.

2C.2.3 Preparation of Recombinant Light Chains from *E. coli*.

The wild type and all mutant human atrial essential light chains were all purified by the same method. The development of this method is described in section 3.2B.1.

Frozen cells (from 1 L of bacterial culture) were thawed in 20-30 ml cell lysis buffer (section 2C.9) and homogenised in a hand homogeniser to disrupt clumps of cells. The cell suspension was then sonicated on ice using a Heat Systems XL2020 Ultrasonic Processor. (Six 15 s pulses at power level 6 were usually sufficient to disrupt the cell walls and to shear the chromosomal DNA). Following sonication, cell debris was removed by centrifugation at 80000g for 30 minutes.

The supernatant was then dialysed into the column buffer (Section 2C.9). The dialysate was centrifuged at 100000g for one hour to remove any insoluble matter and fractionated by ammonium sulphate precipitation. The 40-60% fraction was resuspended in 2 to 3 ml of column buffer and dialysed overnight against this buffer before being applied to a DEAE-fast flow column of bed volume 50 ml. The light chain was eluted with a 0 to 0.4M sodium chloride gradient. Fractions (of size 3 to 4 ml)

containing pure light chain (as judged by SDS-PAGE) were pooled and dialysed extensively into 0.1%(w/v) ammonium hydrogencarbonate containing 0.005% β-mercaptoethanol and then freeze dried. Freeze dried powders were stored at -20°C until required.

2C.2.4 Preparation of *Drosophila* Regulatory Light Chain.

Drosophila RLC was supplied as a powder freeze dried from 1 mM ammonium hydrogencarbonate by our collaborator, Dr David Maughan (University of Vermont, USA). The protein had been expressed and purified from *E. coli* by workers in his laboratory.

2C.2.5 Preparation of human cardiac TnI and TnC.

Recombinant human cardiac TnI (cd TnI) and human cardiac TnC (cd TnC) were prepared according to reported methods (Al-Hillawi *et al.* 1994) and kindly donated by Drs Trayer and Al-Hillawi.

2C.3 Analytical Methods.

2C.3.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis.

Two sorts of SDS-PAGE gels were used. Slab gels were routinely used for monitoring protein purity and integrity and following the results of cross-linking reactions. Stacking gels were used when additional resolution was required. In both cases the samples were made up in the same way.

SDS-PAGE Sample Preparation

Protein solution	120µl
20 % SDS	10 µl
0.1 % Bromophenol Blue	10 µl

β-Mercaptoethanol 5 µl

Samples were boiled for 2 to 5 minutes and spun briefly to collect any condensation. Samples must be alkaline before loading and this is indicated by a blue colour of the sample. If the sample was not alkaline after boiling, 10 to 20 µl of 1 M sodium hydroxide was added. The recipe given above can be scaled down if the amount of material is scarce.

All gels were run in tris/bicine/SDS buffer (section 2C.9) at 30 to 60 mA (constant current).

The composition of 12 % polyacrylamide gel mix and the 3% stacking gel mix is given in section 2C.9. Stacking gels were prepared such that the length of the stack was about 10% of the total length of the gel.

Gels were stained with Coumassie Blue Stain (heated for 5 minutes at 40% power in a microwave) and destained (heated at full power in a microwave for 2 minutes and then shaken at room temperature with fresh destain) until there was little or no background colouration.

2C.3.2 Protein Estimation.

2C.3.2.1 Absorbance Measurements.

Absorbance measurements were routinely used to estimate the concentration of unmodified, purified proteins. The following values were used.

Protein	$A_{280,1mg/ml}$ / $mg^{-1}.ml$	Molecular Mass / kDa
S1(A1)	0.80	112
S1(A2)	0.80	106

Crude S1	0.80	n/a
Myosin	0.57	480
G-actin [(1)]	0.63	42
DNaseI	1.1	31
Rabbit Skeletal A1 ELC	0.22	21
Rabbit Skeletal A2 ELC	0.22	17
Human Atrial ELC (Wild type and N-terminal mutants)	0.22	21
Hm At ELC (A1-11 mutant)	0.22	20
Hm At ELC (A2-like mutant)	0.22	17
Troponin C (human cardiac)	0.2	18
Troponin I (human cardiac)	0.44	24
Drosophila RLC	0.05 [(2)]	24
α-Chymotrypsin	2.0	25
Bovine Serum Albumin (BSA)	0.68	68

Notes: [(1)] Actin was determined at 290 nm.

[(2)] Estimated using GCG program Peptidesort (Devereux *et al.* 1984)

2C.3.2.2 MicroTannin Method for Protein Estimation.

This method was for estimating the concentration of fluorescently labelled proteins and mixtures of proteins. It depends upon the formation of colloidal precipitates of proteins in the presence of tannic acid which are stabilised by gum arabic. The amount of precipitate is related to the amount of protein and is quantified by measuring the light scattering at 520 nm. The assay is sensitive over the range 10 to 60 μg protein (Mejbaum-Katzenellenbogen and Dobryszycka, 1959).

Protein solution (1 ml) was pre-incubated at 25°C for at least 5 minutes before the addition of 1 ml tannin reagent (section 2C.9) and vigorous mixing. The reaction was allowed to proceed for 10 minutes and then 1 ml gum arabic was added. After 5

minutes the A_{520} value was determined. 1 ml of distilled water treated in a similar manner to the protein solutions was used as a blank. Concentrations were determined by comparison to a standard curve, constructed using known concentrations of BSA.

2C.3.3 Protein Sequencing.

2C.3.3.1 Electroblotting onto PVDF Membranes.

Prior to sequencing, proteins were transferred onto PVDF membranes. The protein was separated by SDS-PAGE and the (unstained) gel was placed in the semi-dry blotting apparatus in a sandwich consisting of: two sheets of 2 MM filter paper soaked in anode II buffer (section 2C.9) in contact with the graphite anode, two sheets of 2 MM paper soaked in anode I buffer, the PVDF membrane (pre-soaked in 100 % methanol and rinsed briefly in cathode buffer), the gel and two sheets of 2 MM paper soaked in cathode buffer in contact with the cathode (Matsudaira, 1987). The apparatus was run at 25 mA for 25 minutes and bands visualised by staining with PVDF stain (5 minutes at room temperature) and destained in 50 %(v/v) methanol until background staining was negligible. The membrane was then dried between filter papers and the relevant bands cut out.

2C.3.3.2 N-terminal peptide sequencing.

N-terminal sequencing was carried out by Alta Bioscience using the Edman degradation method (as modified by Findlay and Geisow, 1989) on an Applied Biosystems 473A automated protein sequencer.

2C.3.4 Mass Spectrometry

Mass spectrometry was used to verify the molecular masses of expressed proteins. All mass spectra were run on a Kratos Kompact MALDI 3 matrix assited laser desorption machine by Mr P Ashton, School of Chemistry, The University of Birmingham. This

technique (Hillenkamp and Karas, 1990; Jardine, 1990; Biemann, 1990) is capable of yielding high quality data, but is highly dependant on sample quality.

2C.4 PEPTIDE SYNTHESIS & PURIFICATION.

2C.4.1 Peptide Synthesis.

Peptides were synthesised by Alta Bioscience using the Merrifield solid phase method (Atherton and Sheppard, 1989) and were supplied as freeze dried powders.

2C.4.2 Peptide Purification.

All peptides used in this study were purified by Miss Sue Brewer by HPLC. They were stored as freeze dried powders at -20°C or frozen in solution until required.

2C.4.3 Peptide Sequences.

Most of the peptides used in this study had been synthesised previously for use in NMR experiments.

Rabbit Skeletal A1 Light Chain 1-32

Amine- A-P-K-K-D-V-K-K-P-A-A-A-A-A-P-A-P-A-P-A-P-A-P-A-P-A-P-A-K-P-K-E-Amide

Rabbit Skeletal A1 Light Chain 1-10

Amine-A-P-K-K-D-V-K-K-P-A-Carboxyl

Drosophila RLC 1-21

Amide-A-D-E-K-K-K-V-K-K-K-K-T-K-E-E-G-G-T-S-E-T-Carboxyl

2C.5 HYBRIDISATION OF EXPRESSED LIGHT CHAINS INTO S1.

All hybridisations were carried out using a five-fold molar excess of ELC over S1 in a total volume of 5 ml at 4°C. The method is based on that of Wagner and Weeds, 1977 but using a more alkaline pH (Ueno and Morita, 1984) and separating the isoenzymes on SP-Trisacryl (Trayer and Trayer, 1988). Generally ELCs which were A1-like in sequence were hybridised into S1(A2) and those that were A2-like into S1(A1). The hybridisation mixture contained:

		Final concentration
Solid Ammonium Chloride (1.25 g)	0.875 ml	4.7 M
0.1 M TEA.EDTA, pH 8.0	0.10 ml	2 mM
0.1 M DTT	0.10 ml	2 mM
Concentrated Ammonia Solution	0.25 ml	approx. 0.9 M
S1	X ml	
25 mM TEA.HCl, pH 8.0	(3.675 - X) ml	

S1 was used directly as stored (ie in the presence of sucrose). All the components, except S1 were mixed and the light chain added as freeze dried powder. When the light chain had dissolved, S1 was added and the mixture stirred for 20 minutes. After this time, the mixture was applied to a gel filtration column (Sephadex G25, 50 ml, pre-equilibrated in S1 separation buffer, see section 2C.9) to remove the ammonium chloride. Fractions containing protein and no salt were identified by monitoring the A_{280} value and the conductivity. These fractions were pooled and applied to an SP-tris acrylamide column (70 ml, pre-equilibrated in S1 separation buffer). After unbound material (mainly mixed light chains) had washed through, bound material was eluted with a 0 to 0.2 M sodium chloride gradient. Fractions containing pure S1 isoenzymes were identified by SDS-PAGE (section 2C.3.1) and pooled. They were subsequently concentrated by precipitation in 70 % ammonium sulphate (section 2C.1.2) and then resuspended in 0.5 to 1.0 ml 5 mM TEA.HCl, pH 7.5, 0.25 mM DTT and dialysed

overnight. Before use, the solutions were clarified by centrifugation at 15000g for 15 minutes.

2C.6 DISCONTINUOUS MGATPASE ASSAYS.

Actin activated MgATPase assays were carried out by the discontinuous assay procedure (Winstanley *et al.* 1979) - except in this case the ionic strength was kept low to magnify the difference between S1(A1) and S1(A2)-like kinetics. All assays were carried out in buffer containing 5 mM TEA.HCl, pH 7.5 at 25°C.

Actin was dissolved in buffer A (Section 2C.9) and dialysed vigorously overnight into this buffer. Insoluble material was pelleted by centrifugation at 80000g for 30 minutes and the resulting G-actin solution polymerised to F-actin by the addition of magnesium chloride to a final concentration of 2 mM. To keep the ionic strength constant following the addition of F-actin in buffer to the assay mix, compensating amounts of buffer A, plus 2 mM magnesium chloride was added such that the final volume of buffer A was always 40 µl per ml of assay mix. S1 concentration varied between 0.10 and 0.16 µM.

Reactions were initiated by the addition of MgATP such that the final Mg^{2+} concentration was 2.5 mM and the final ATP concentration was 2 mM. Care was taken to ensure that no more than 30% of the ATP was used up in each reaction. For each set of reactions a control was set up with no actin to correct for the non-actin activated MgATPase of S1 and any residual phosphate. Reaction rates were calculated from the amount of inorganic phosphate released during the course of the reaction (determined spectrophotometrically (Fiske and Subbarow, 1925), the zero actin control being used as a blank). Activities were calculated by dividing the rates (in µmol phosphate released s^{-1}) by S1 concentration. Data were analysed by non-linear curve fitting to the equation Activity = $(k_{cat}[Actin])/(K_M+[Actin])$ using the package Fig-P for Windows (Biosoft, Cambridge, UK). All points were weighted equally.

2C.7 CROSS-LINKING.

2C.7.1 Cross-Linking Using EDC.

The procedure used for cross-linking was modified after (Andreev and Borejdo, 1995). Freeze dried light chains were reconstituted in 5 mM TEA.HCl pH 7.5, 0.25 mM DTT in the presence of 1 mg DTT per mg freeze dried powder and 10 µl 1 M sodium hydroxide per ml buffer, and dialysed overnight against this buffer. Actin was reconstituted as described in section 2C.6. The proteins were mixed in different molar ratios (see results chapters) such that the total volume was 50 µl, and incubated at room temperature for one hour. After this time, EDC was added to a final concentration of 0.24 M (ie 10µl of 0.4 M giving a total volume of 60 µl), the reaction mixed gently and allowed to proceed for a further hour. At this point the reaction was stopped by making the total volume into an SDS-PAGE sample and the products separated by SDS-PAGE (section 2C.3.1).

2C.7.2 Glutaraldehyde Cross-Linking of F-actin.

G-actin solution (about 1 $mg.ml^{-1}$ in buffer A) was prepared as described in section 2C.6. The G-actin solution was polymerised to F-actin by the addition of magnesium chloride to 2 mM and sodium chloride to 50 mM. Freshly made glutaraldehyde solution (section 2C.9) was added to give a final concentration of 20 mM and the reaction incubated at 25°C for 30 minutes. Cross-linking was terminated by the addition of sodium metabisulphate (section 2C.9) to a final concentration of 50 mM. The cross-linked product was then dialysed overnight against 10 mM TEA.HCl, pH 7.5, 0.1 M sodium chloride, 0.1 M magnesium chloride, 0.1 mM ATP. Successful cross-linking was confirmed by SDS-PAGE (section 2C.3.1).

2C.8 Covalent Modification of Proteins.

2C.8.1 Fluorescent Labelling of A1 Light Chain and Actin.

A1 light chain and actin labelled with 1,5-IAEDANS (figure 2.4a) were prepared according to Trayer and Trayer, 1988 and kindly donated by Dr HR Trayer.

Briefly, G-actin (between 3 and 6 $mg.ml^{-1}$) in buffer A was labelled with a twenty-fold excess of label in the dark for 18 hours. The probe was then removed by gel filtration on Sephadex G25 and dialysis against buffer A and the labelled protein freeze dried.

High concentrations (greater than 60 $mg.ml^{-1}$) of A1 ELC were dissolved in 0.5 M TEA.HCl, pH 8.0, 6 M guanidine hydrochloride, 2 mM EDTA, 130 mM DTT and left to reduce at room temperature overnight. The DTT concentration was reduced by passage down a Sephadex G25 column equilibrated in the same buffer but with 2 mM DTT. A twenty five-fold excess of label was added and the reaction mix left stirring, in the dark at room temperature for about 20 hours. Excess probe and denaturant were removed by gel filtration on Sephdex G25 pre-equilibrated in 25 mM TEA.HCl, pH 8.0, 0.5 mM DTT. The labelled light chain was then dialysed extensively into 1 %(w/v) ammonium hydrogencarbonate / 20 mM β-mercaptoethanol before freeze drying.

2C.8.2 Biotinylation of Actin.

2C.8.2.1 NHS-Biotinylation

N-hydoxysuccinimidobiotin can be used to label proteins on exposed amino groups (ie ε-amino groups of lysine residues and the N-terminus). The structure of this reagent is shown in Figure 2.4b.

Actin labelled in this manner was obtained from Cytoskeleton, Denver, CO, USA, supplied frozen in 2 mM tris.HCl, pH 8.0, 0.2 mM calcium chloride, 0.2 mM ATP, 0.5 mM DTT and stored at -70°C until required. The manufacturers claim an average labelling of 1 molecule biotin per molecule actin and that the protein has an activity (assayed by its ability to polymerise) comparable to unlabelled.

*2C.8.2.3 Labelling with 3-(*N-*Maleimido-propionyl) Biocytin.*

Maleimido groups can be used to irreversibly label thiol groups in proteins. The reaction does not involve a change in the redox state of the thiol group is not, therefore reversed by thiol reducing agents such as DTT. 3-(*N*-Maleimido-propionyl) Biocytin (MPB) (figure 2.4c) consists of biotin conjugated to a lysine residue and a short aliphatic spacer (Bayer *et al.* 1985).

Actin was reconstituted in buffer A as described in section 2C.6 and labelled by stirring with a six-fold molar excess of MPB for 24 hours. After this time, free MPB was removed by gel filtration on Sephadex G25 (pre-equilibrated in buffer A).

The degree of biotinylation was estimated by mixing a sample of the actin solution of known concentration with streptavidin-sepharose, shaking gently at room temperature for one hour, removing the resin by centrifugation at 15000g for 10 minutes and determining the amount of protein remaining in the supernatant.

2C.9 BUFFERS & OTHER SOLUTIONS.

Myosin Filament Buffer

Disodium Hydrogenphosphate	14 mM
Sodium Dihydrogenphosphate	6 mM
Sodium Chloride	120 mM
Sodium EDTA	1 mM
DTT	0.25 mM

The final pH is 7.2 and the total phosphate concentration 20 mM.

S1 Separation Buffer

MOPS	10 mM

The solution was adjusted to pH 7.5 with 5 M sodium hydroxide.

Buffer A

TEA	5 mM
Calcium Chloride	0.2 mM
ATP	0.2 mM
DTT	0.25 mM

The solution was adjusted to pH 7.5 with concentrated hydrochloric acid.

Cell Lysis Buffer

TEA	25 mM
Sodium Chloride	150 mM
Sucrose	250 mM
EDTA	2 mM
EGTA	2 mM
DTT	0.25 mM

The solution was adjusted to pH 7.5 with concentrated hydrochloric acid.

Column Buffer (for recombinant ELC prep.s)

TEA	25 mM
EDTA	2 mM
DTT	0.25 mM

The solution was adjusted to pH 7.5 with concentrated hydrochloric acid and supplemented with sodium chloride as required.

PMSF

70 mg PMSF dissolved in 1 ml dry ethanol gives a 400 mM solution. 0.23 ml of this solution per litre of buffer gives a solution of approximately 100 μM.

Tris/Bicine/SDS PAGE Buffer

Tris	0.1 M
Bicine	0.1 M
SDS	0.1 %(w/v)

12 % PAGE mix.

Per 10 ml:

Water	6.0 ml
50 %(w/v) Bis/Acrylamide	2.4 ml
1 M Tris/Bicine	1.0 ml
20 %(w/v) SDS	50 μl
TEMED	50 μl
10 %(w/v) APS	100 μl

50 %(w/v) Bis/Acrylamide is made by dissolving 1 g per litre N,N'-methyl-*bis*-acrylamide and 49 g per litre acrylamide in distilled water.

3% Stacking Gel Mix

Per 10 ml:

Water	3.2 ml
0.5 M Tris.HCl, pH 6.9	6.0 ml
50 %(w/v) Bis/Acrylamide	1.2 ml
20 %(w/v) SDS	50 μl

TEMED	50 µl
10 %(w/v) APS	100 µl

Coomassie Blue Stain

Page Blue-83	0.4 %(w/v)
Methanol	46 %(v/v)
Acetic Acid	8 %(v/v)

SDS-PAGE Destain

Acetic Acid	14 %(v/v)
Ethanol	10 %(v/v)

Bromophenol Blue

Bromophenol Blue	0.1 %(w/v)
Sucrose	approximately 10 %(w/v)

Tannin Reagent

490 ml of 1 M hydrochloric acid was warmed to approximately 80°C and 10 ml of melted phenol was added. 50 g of tannic acid was added and the solution mixed well. When cool the mixture was filtered.

Gum Arabic

1 g of gum arabic was dissolved in 500 ml of warm water and the resulting solution filtered when cool.

Anode Buffer I

Tris	0.3 M
Methanol	10 %(v/v)

The unadjusted pH was 10.4.

Anode Buffer II

Tris	25 mM
Methanol	10 %(v/v)

The unadjusted pH was 10.4.

Cathode Buffer

Tris	25 mM
Methanol	20 %(v/v)

The solution was adjusted to pH 9.4 with concentrated hydrochloric acid.

PVDF Stain

PAGE Blue	0.2 %(w/v)
Methanol	50 %(v/v)
Acetic Acid	1 %(v/v)

Glutaraldehyde Solution

Glutaraldehyde	1 M (ie 1 in 5 dilution of 5 M stock)

The solution was adjusted to pH 7.5 with 0.1M sodium hydroxide.

Sodium Metabisulphate Solution

Sodium Metabisulphate	0.5 M
Potassium Dihydrogenphosphate	0.01 M

The solution was adjusted to pH 7.5 with 5 M sodium hydroxide.

2D SPECIALIST METHODS.

2D.1 SURFACE PLASMON RESONANCE.

2D.1.1 Physical Principals.

When light passes from glass into air two physical processes occur. Some of the light is reflected at the interface and some passes out and is refracted (the ray of light bends towards the normal) in accordance with Snell's Law (Feynman, 1964). When the angle of incidence of the light on the interface is greater than the critical angle (the angle for which the angle of refraction would be 90°) refraction is no longer possible and "total" internal reflection occurs.

Total internal reflection is, however, not total. The intensity of the incident ray is slightly greater than the intensity of the reflected ray (ie there are more photons in the incident ray than in the reflected one), but the frequency of the light is unchanged (ie the energy of individual photons is unaltered). This effect is normally trivial, but can be enhanced if a thin metal layer is placed at the interface (Kretschmann and Raether, 1968; Hummel, 1985; Bube, 1988). Metals have "pools" of relatively mobile electrons which are not constrained by association with any particular atom (this is why they are good conductors of heat and electricity); among inert metals, silver and gold have particularly mobile electron pools (Feynman, 1964). These electrons can be made to oscillate at the same frequency as the incident light by the absorption of photons. Electrons oscillating in this way are referred to as plasmons. At one angle, the resonance angle, coupling between the oscillation of the photons and the electrons in the metal layer is most efficient and the greatest drop in the intensity of the reflected ray is seen (Otto, 1968).

The resonance angle depends on (among other factors) the electrical permittivity (ε) and, consequently, the refractive index (n) of the medium immediately beyond (up to about 3 times the wavelength of the incident light) the metal layer. It should be noted that no light passes beyond the interface and therefore the reflected ray is not affected

by the absorption characteristics of the material beyond the metal layer. The dependence of the resonance angle on ε can be demonstated experimentally. If a beam of light undergoing total internal reflection at a glass surface coated with a thin layer of silver is moved through a range of incident angles then the resonance angle can be determined by measuring the intensity of the reflected ray. The intensity will be at a minimum at the resonance angle. If the refractive index of the medium beyond the metal layer is varied (eg by changing from acetone, n=1.36 to hexane, n=1.42) then the resonance angle changes (Mayo and Hallock, 1989) (figure 2.5). Alternatively, a similar experimental setup can be used to monitor mass building up on the surface. If macromolecules (eg proteins) are adsorbed onto the metal layer then the bulk refractive index of the medium will change. If the molecules are all of the same type, then they will all have the same electrical properties and the bulk refractive index change and hence the change in the resonance angle will be proportional to the mass deposited. Early applications of surface plasmon resonance to the study of macromolecular interactions involved the direct adsorption of proteins onto metal surfaces. For example, the adsorption of IgG onto gold surfaces and the binding of anti-IgG was monitered by measuring the change in the resonance angle as mass accumulated on the surface as a result of binding (Geddes *et al.* 1994) and human serum albumin was adsorbed onto silver and the binding of anti-HSA was detected in a similar fashion (Flanagan and Pantell, 1984; Silin *et al.* 1993). This approach has two serious flaws: first only a limited number of proteins can be adsorbed onto metal surfaces without loss of function and second, in order to obtain kinetic data, it is necessary to moniter changes in the resonance angle as they occur. Both these problems were addressed in the development of the BIA range of machines by Pharmacia Biosensor.

2D.1.2 Implementation in BIAcore™ 2000.

The BIAcore 2000 machine incorporates a number of technical features which enable the practical use of surface plasmon resonance in the study of macromolecular interactions.

The problems of direct adsorption to the metal layer is overcome by coupling a carboxymethyldextran layer to the metal surface. This provides a hydrophillic layer with functional groups to which proteins etc can be coupled covalently (Löfås and Johnsson, 1990). The problem of monitoring changes in the resonance angle as they occur is overcome by using a wedge shaped beam of light (figure 2.6) and a diode array detector which monitors the intensity of light over a range of angles of reflection simultaneously. In addition the machine includes sophisticated liquid handling to enable the delivery of solutions to the surfaces and software for the analysis of kinetic data.

A typical BIAcore 2000 experiment requires that one component of the system be immobilised onto the carboxymethyldextran surface (see 2D.1.3) and the other is passed over it. The experiment consists of two phases: an "association" phase and a "dissociation" phase. (Although these names are in fact inappropriate since both association and dissociation occur in both phases (Fisher and Fivash, 1994)). During the association phase the component in solution (the analyte) is passed over the immobilised molecule (the ligand) and the resonance angle (reported as resonance units - RU - where 1 RU is equivalent to a resonance angle change of 0.1° (O'Shannessy *et al.* 1994)) shifts due to the accumulation of mass at the surface. The system is a continuous flow one, and so the concentration of free analyte remains constant over the course of the association phase. The dissociation phase is initiated by switching the flow to buffer alone; analyte bound to immobilised ligand dissociates and the resonance angle moves back towards the original value (O'Shannessy *et al.* 1994; Panayotou *et al.* 1993; Raghavan and Bjorkman, 1995; Fisher and Fivash, 1994; Szabo *et al.* 1995).

From these two phases, kinetic rate constants describing the association and dissociation reactions can be derived. Non-linear curve fitting is the preferred method for determining these constants (O'Shannessy *et al.* 1993). Equilibrium constants can then be derived from the rate constants.

2D.1.3 Methodology

Proteins were immobilised using available immobilisation chemistries according to the instructions given by the manufacturer. A summary of the methods used is given in Figure 2.7. Data were analysed using the software provided by the manufacturer (BIAevaluation) which uses non-linear curve fitting to derive kinetic constants. All buffers were filtered and degassed before use.

2D.1.3.1 Amine coupling.

Proteins were immobilised onto the CM dextran surface using NHS/EDC chemistry. A 7 minute pulse of 0.05 M NHS/0.2 M EDC was used to derivatise the surface, proteins injected over the dervatised surface for varying contact times and the surface deactivated by a 4 minute pulse of 1 M ethanolamine hydrochloride, pH 8.5. In addition to blocking unreacted ester groups on the surface, the relatively high ionic strength of this solution helps remove non-covalently bound material from the surface. This method attaches proteins through exposed amine groups (lysine side chains and unblocked N-termini) and thus there is the potential for a variety of different orientations of immobilised ligand to be presented to soluble analytes.

2D.1.3.2 Thiol coupling.

Thiol coupling attaches proteins through accessible thiol groups. Attachment is through a bifunctional reagent (2-(2-pyridinyldithio)ethaneamine hydrochloride, PDEA) which contains both amine and thiol groups. Following NHS/EDC derivatisation of the surface as described above (section 2D.1.3.1), 80 mM PDEA freshly dissolved in 0.1 M borate buffer, pH 8.5 was injected for 7 minutes. Protein (dissolved in buffer lacking DTT or any other similar thiol reagent) was passed over the immobilised PDEA to allow disulphide bonds to form between the PDEA and protein. Remaining thiol sites on the surface were blocked with 50 mM cysteine/1 M sodium chloride freshly dissolved in 100 mM sodium formate buffer, pH 4.3.

2D.1.3.3 Streptavidin-biotin coupling.

Proteins derivatised with biotin (as described in section 2C.8.2) were passed over CM-dextran surfaces which had been purchased with streptavidin immobilised on the surface. The extremely high affinity between biotin and streptavidin means that proteins captured by this system are effectively permanently bound to the surface.

2D.2 *IN VITRO* MOTILITY ASSAYS.

In vitro motility assays were carried out in collaboration with Drs John Sparrow and Justin Molloy (Department of Biochemistry, University of York, UK) essentially after the method of (Anson *et al.* 1995).

Myosin fragments (HMM and S1 isoenzymes) were immobilised on clean, glass cover-slips that had been previously coated with 0.1 %(w/v) nitrocellulose in amyl acetate. Immobilisation was allowed to proceed for between 60 and 90 seconds and was followed by blocking of the remaining binding sites on the cover-slip with 0.5 $mg.ml^{-1}$ BSA in AB- buffer (the composition of buffers etc is given below) for a further 60 to 90 seconds. Rhodamine-phalliodin stabilised F-actin (approximately 1/200 dilution in AB-/BSA/GOC/DTT buffer of a 4 $mg.ml^{-1}$ stock) was then flowed over the surface. Filaments were viewed under an inverted fluorescent microscope and the rhodamine phallodin fluorescence excited using the Nikon green filter. They were observed using an EEV photon intensified CCD camera and experiments recorded using a Panasonic Video Plus 4 NVSD40 recorder linked to a black and white monitor. The experimental set-up is shown in Figure 2.8.

Filament movement was initiated by the addition of AB+/BSA/GOC/DTT buffer. “Dead heads” (ie those whose binding to actin cannot be reversed by the addition of MgATP) were neutralised by the addition of an excess of unlabelled F-actin (0.25 $mg.ml^{-1}$ in AB+/BSA/GOC/DTT buffer). Fields of view were recorded until photobleaching reduced the phallodin fluoresence to background levels.

Data were analysed using the software package Retrac 1.15 (University of York) running on an IBM compatible 486 PC linked to a Panasonic AG7350 video recorder

and a Panasonic TC1470Y monitor. Student's t-test was used to assess whether data sets differed significantly from each other.

AB- Buffer:

Imidazole-HCl	25 mM
Potassium chloride	25 mM
Magnesium chloride	4 mM
EGTA	1 mM

pH to 7.4 with 1 M hydrochloric acid.

AB+ Buffer:

As AB- buffer with 2 mM ATP.

AB-/BSA Buffer:

As AB- with 0.5 $mg.ml^{-1}$ BSA

AB-/BSA/GOC/DTT Buffer:

As AB- but with:

DTT	20 mM
Catalase	0.02 $mg.ml^{-1}$
Glucose oxidase	0.1 $mg.ml^{-1}$
Glucose	3 $mg.ml^{-1}$

AB+/BSA/GOC/DTT Buffer:

As AB-/BSA/GOC/DTT buffer, except AB+ was used in place of AB-.

2D.3 COMPUTING RESOURCES FOR DNA & PROTEIN SEQUENCE ANALYSIS.

DNA and protein sequence analysis was carried out using the GCG suite of programs (Devereux *et al.* 1984) (versions 7.0 and 8.0) running on a VAX cluster operated by the University of Birmingham Information Services.

Chapter Three:

The Rôle of the N-terminal Extension of A1-type ELCs in Modulating Myosin Motor Function.

3.1 INTRODUCTION.

There is considerable circumstantial evidence that the N-terminus of A1-type myosin essential light chains binds actin (Henry *et al.* 1985b; Trayer *et al.* 1987; Milligan *et al.* 1990; Trayer and Trayer, 1985; Trayer *et al.* 1977; Winstanley *et al.* 1977; Sutoh, 1982; Labbé *et al.* 1986; Andreev and Borejdo, 1995 and see section 1.4.5). This actin binding event is assumed to result in the kinetic modulation that this ELC isoform confers on the myosin heavy chain. However, this connection has never been proven, nor has the actin binding site been located precisely within the 40-odd residue extension seen in A1-type ELCs.

The actin binding site was located by a protein engineering approach: deletion mutants lacking the first 11 and first 45 residues were constructed and assayed for their ability to bind actin and to modulate the kinetics of S1. Of course a necessary prerequisite of these studies was the expression of a wild type A1-type ELC (the HmAtELC) in a suitable host (*E. coli*) and to develop a purification strategy for the recombinant protein. Furthermore, an attempt was made to quantify the ELC-actin interaction using a surface plasmon resonance based biosensor.

Our ability to assess the consequences of mutations in the ELC would be enhanced if the effects could be monitored in an *in vitro* motility assay. In order to make this a viable technique it was necessary to establish conditions under which the S1 isoenzymes - S1(A1) and S1(A2) - could be reproducibly distinguished.

3.2A PAPER I: "THE N-TERMINUS OF A1-TYPE MYOSIN ESSENTIAL LIGHT CHAINS BINDS ACTIN AND MODULATES MYOSIN MOTOR FUNCTION."

The following paper has been submitted to the journal Biochemistry and is currently undergoing review.

The N-terminus of A1-type Myosin Essential Light Chains Binds Actin and Modulates Myosin Motor Function.[+]

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Running title: N-terminus of A1-type Myosin ELC binds Actin.

[1]Abbreviations: ELC, myosin essential light chain; RLC, myosin regulatory light chain; S1, myosin subfragment 1 prepared by proteolytic digestion; S1(A1), S1(A2), rabbit skeletal myosin subfragment 1 produced by chymotryptic digestion and containing either the alkali 1 (A1 or LC1) or the alkali 2 (A2 or LC3) ELC; HmAtELC, human atrial ELC; HmAtELCΔ(1-11), a deletion mutant of HmAtELC missing the first 11 amino acids; HmAtELCΔ(1-45), a deletion mutant of the HmAtELC missing the first 45 amino acids; F-actin, polymeric actin; G-actin, monomeric actin; DNase-I, deoxyribonuclease I; IPTG, isopropyl-1-thio-β-D-galactopyranoside; DTT, dithiothreitol; TEA, triethanolamine; PMSF, phenylmethylsuphonyl fluoride; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; 1,5-IAEDANS, N-(iodoacetyl)-N′-(1-sulpho-5-napthyl)ethylenediamine; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PCR, polymerase chain reaction; MOPS, 3-(N-morpholino)propane-sulphonic acid.

ABSTRACT. The existence of two isoforms (A1 and A2) of the myosin essential light chain (ELC) results in two isoenzymes of myosin subfragment 1 (S1) - S1(A1) and

S1(A2). The two isoenzymes differ in their kinetic properties with S1(A1) having a lower apparent K_M for actin and a slower turnover of MgATP (k_{cat}) than S1(A2). The two forms of the ELC differ only at their N-termini where A1 has an additional 40 or so amino acids that are not present in A2.

Using expressed, recombinant human atrial myosin essential light chain (an A1-type ELC) we have initiated a programme of protein engineering to determine the role of this N-terminal extension in modulating the kinetics of the myosin motor. The wild type protein was readily expressed in *Escherichia coli* and purified by ammonium sulfate fractionation and ion exchange chromatography. The recombinant light chain is biologically active - as shown by hybridising it into rabbit skeletal S1 and determining the actin-activated MgATPase kinetics which are similar to those for rabbit skeletal S1(A1) under the same conditions Deletion of the first 45 amino acid residues results in an ELC similar to the rabbit skeletal A2 isoform and, when hybridized into rabbit skeletal S1, in kinetic properties similar to S1(A2). Similar results are obtained with an ELC mutant that lacks the first eleven residues, localising the actin binding site to this region of the molecule.

Using the zero-length cross-linker EDC, we were able to cross-link the wild type human atrial ELC (both hybridized into S1 or "free" in solution) to F-actin. Neither deletion mutant cross-links, thus confirming that the first eleven residues encodes an actin binding site and that this interaction results in kinetic modulation of the myosin motor. Furthermore, while free A1-type ELCs cross-link to both polymeric F-actin and monomeric G-actin:DNase-I complex, the same ELCs hybridized into S1(A1) only cross-links to F-actin. This suggests that the light chain binds to a different actin monomer than the heavy chain.

INTRODUCTION. Muscles vary greatly in the demands made of them. One way that organisms can respond to these demands and adapt to new ones is to express different isoforms of the contractile proteins. The role of myosin heavy chain isoforms in this adaptation is well established (Galler et al. 1994; Bottinelli et al. 1994b; Moss et al. 1995) and, recently, the involvement of essential light chain (ELC)[1] isoforms has also

been shown (Bottinelli et al. 1994a). Their role appears to be in "fine tuning" the heavy chain. Against a background of constant heavy chain composition, shortening velocity will be determined by the ELC isoform content. By combining the various heavy and light chain isoforms, almost continual variation in the contractile properties of muscle fibres is possible (Schiaffino & Reggiani, 1994).

Myosin is a molecular motor of molecular mass 480 kDa consisting of two heavy chains (200 kDa each), two regulatory light chains (RLC, 20 kDa) and two ELCs (18-20 kDa). Its function is to couple the energy released on the hydrolysis of MgATP with the mechanical work of pulling past its partner protein, actin. The crystal structure of myosin subfragment 1 (S1) - a soluble proteolytic fragment which retains the motor functions of the intact molecule (Lowey et al. 1969) - has been solved (Rayment et al. 1993b) and shows two light chains (one ELC and one RLC) per S1 clamped sequentially around an 8 nm stretch of α-helical heavy chain. Undoubtedly one of the main functions of the light chains is to stabilize this structure allowing it to act as a "lever arm" amplifying small structural changes at the active site to give rise to a larger power stroke (Reedy, 1993; Uyeda et al. 1996). However, this region can also be a site of regulation. In scallop muscle, calcium ions bind directly to the ELC and this event switches on the motor (Szent-Györgyi et al. 1973). In smooth muscle, phosphorylation of the RLC (controlled by a Ca^{2+}-calmodulin dependant kinase) controls filament formation and MgATPase activity (reviewed in Allen & Walsh, 1994).

In striated muscle, calcium sensitivity (and hence regulation) is conferred by the troponin/tropomyosin complex located on the actin filament. However, there are two forms of ELC - termed A1 (or LC1) and A2 (or LC3). In fast skeletal muscle both forms occur, but in slow skeletal and cardiac muscle only A1-type ELCs are found. The existence of two ELC isoforms in fast skeletal muscle gives rise to two S1 isoenzymes - S1(A1) and S1(A2) - depending on which ELC is associated with the heavy chain. The two isoenzymes have different kinetic properties (Weeds & Taylor, 1975; Wagner & Weeds, 1977; Pope et al. 1981). In general, S1(A1) has a lower apparent K_M for actin and a lower k_{cat} than S1(A2), suggesting that S1(A1) has a higher affinity for actin, but works more slowly. This result is borne out by *in vitro* motility assays where whole

myosin carrying only A1-type ELCs moves actin more slowly than that carrying only A2-type ELCs (Lowey et al. 1993). Furthermore in whole fast skeletal muscle fibres (containing only one heavy chain isoform), the unloaded shortening velocity correlates to the amount of A1 in the fibre (Bottinelli et al. 1994a): the greater the amount of A1, the slower the phenotype. It appears that while heavy chain isoform is the principal determinant of a muscle's contractile properties, the ELC isoform acts to fine tune this - providing a range of contractile speeds (Schiaffino & Reggiani, 1994).

The only difference between A1 and A2-type ELCs is at the N-terminus where A1 has an additional 40 or so amino acids (42 in rabbit fast skeletal muscle (Frank & Weeds, 1974), Figure 1). The two proteins are coded by the same gene and result from differential splicing of the same mRNA transcript (Nabeshima et al. 1984). It thus stands to reason that any difference between the two isoforms will result from this N-terminal extension in A1-type ELCs. The extreme N-terminus (the first 9 residues in HmAtELC) is rich in lysine residues and the remainder of the extension is rich in (Xxx-Pro) repeats.

The lower apparent K_M for actin of S1(A1) suggests that this isoenzyme binds more strongly to actin. This conclusion is supported by F-actin affinity chromatography: The two isoenzymes can be separated in this manner, with S1(A2) eluting before S1(A1) (Winstanley et al. 1977). A number of investigators have shown that A1 in S1(A1) can be covalently cross-linked to F-actin using zero-length cross-linkers (Sutoh, 1982; Labbé et al. 1986; Andreev & Borejdo, 1995). However, attempts to cross-link the free light chain to actin met with failure (Labbé et al. 1986). Electron microscopy image reconstruction of F-actin decorated with S1(A1) shows electron density close to actin which is not present when S1(A2) is used (Milligan et al. 1990; Waller et al. 1995). NMR studies show that resonances attributed to amino acids in the N-terminus of A1 are specifically broadened on the addition of F-actin (Henry et al. 1985a; Henry et al. 1985b; Trayer et al. 1987). All this suggests, but does not prove, that the increased affinity of S1(A1) for F-actin compared to S1(A2) results from a direct interaction between the N-terminal extension of A1 and actin.

We used a protein engineering approach to address this problem. Following successful bacterial overexpression and subsequent purification of the HmAtELC (an A1-type ELC (Arnold et al. 1988)), we showed by deletion mutagenesis that the first 11 (or fewer) amino acid residues are required for modulation of the MgATPase kinetics. In contrast to previous reports, we were able to cross-link the free ELC and actin, but only the wild type protein interacted. Thus, we were able to establish, for the first time, a correlation between actin binding by the ELC and modulation of the MgATPase kinetics.

If A1-type ELCs contain an actin binding site, it is important to ask the question whether it binds to the same actin monomer as the heavy chain or a different one. We addressed this problem by comparing the cross-linking of this ELC with polymeric F-actin and the monomeric G-actin:DNase-I complex. The heavy chain can be cross-linked to both; whereas A1 in S1(A1) can only be cross-linked to F-actin but free light chain also cross-links to both. This suggests that the heavy and light chains bind to different actin monomers in the actin filament - most likely monomers immediately adjacent to each other.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotide primers were purchased from AltaBioscience, University of Birmingham. Restriction enzymes, T4 DNA ligase and broad range protein molecular mass markers were from New England Biolabs, Hertfordshire, UK. Taq polymerase was bought from Boeringer, Mannheim, Germany. pET-11c expression vector was from AMS Biotechnology. Bacterial growth media were from Difco, Surrey, UK. Resins for ion-exchange chromatography were bought from Pharmacia, Hertfordshire, UK. DNase I was from Worthington Biochemicals, New Jersey, USA. All other reagents were purchased from Sigma Chemical Co., Poole, UK.

Construction of wild type and mutant expression vectors. cDNA encoding the full length atrial essential light chain (Arnold et al. 1988) in the vector pUC-19 was a generous gift from Dr Anna Starzinski-Powitz (Cologne). The wild type sequence was amplified using the polymerase chain reaction (PCR) (Saiki et al. 1988). PCR primers

were designed so as to introduce an *NdeI* restriction enzyme site 5' to the start of the gene and a *BamHI* site 3' to the stop codon. The PCR product was digested with *NdeI* and *BamHI* and ligated into *NdeI*/*BamHI* cut pET-11c (Studier et al. 1990; Studier & Moffat, 1986) using T4 DNA ligase. The use of *NdeI* which contains the sequence ATG in its recognition site permits the cloning of the gene in frame and with a start (methionine) codon at the 5' end.

N-terminal deletion mutants were constructed using the same 3' PCR primer as the wild type with 5' primers designed to select the appropriate start site. HmAtELCΔ(1-11) started at Ala-12 and the HmAtELCΔ(1-45) mutant started at Asp-46 (Figure 1). The former mutant does not contain the proposed actin binding site and the latter is equivalent to the rabbit skeletal A2 light chain. Again, 5' primers incorporating an *NdeI* restriction site were used. All constructs were verified by double stranded DNA sequencing.

Expression of wild type and mutant proteins. Expression vectors carrying the appropriate sequence were transformed into competent *E. coli* BL21(DE3) (Studier & Moffat, 1986), using standard procedures (Sambrook et al. 1989). Single colonies were picked and grown overnight in 40 ml of Luria Broth (Sambrook et al. 1989) supplemented with 100 $\mu g.ml^{-1}$ ampicillin (for selection) at 37°C. These overnight cultures were diluted into 500 ml of NZCYM medium (Sambrook et al. 1989), pre-warmed to 37°C, supplemented with 100 $\mu g.ml^{-1}$ ampicillin. These cultures were grown, shaking at 37°C until A_{600} exceeded 1.0 (typically two to three hours). Cultures were then induced by addition of IPTG to a final concentration of 0.4 mM, and then grown for a further two to five hours. After this time cells were harvested by centrifugation at 3000g and frozen at -70°C. Expression was monitored by comparing SDS-PAGE of cell extracts before and after induction and judged successful if an additional band of approximately correct molecular mass appeared after induction.

Purification of actin and myosin. All protein manipulations were carried out at 4°C, unless otherwise stated. Myosin S1 and actin were prepared from rabbit skeletal muscle. Actin was prepared according to the method of Spudich and Watt (Spudich &

Watt, 1971) and stored at -20°C as a freeze dried powder. Myosin S1 was prepared by chymotryptic digestion (which produces S1 lacking the RLC binding site but with an intact ELC and ELC binding site) (Weeds & Taylor, 1975). The isoenzymes - S1(A1) and S1(A2) - were resolved on an SP-Trisacryl M™ (IBF Biotechnics, France) column (Trayer & Trayer, 1988). Aliquots were shell frozen in the presence of 4 mg sucrose per mg S1 and 2 mM DTT and stored at -20°C. Rabbit skeletal A1 light chain was prepared by urea dissociation from myosin (Henry et al. 1985a) and was a gift from Dr Hylary Trayer. Actin and rabbit skeletal A1 light chain were labelled with 1,5-IAEDANS as described by Trayer & Trayer, 1988.

Preparation of recombinant proteins. Frozen cells (from 1 L of bacterial culture) were thawed in 20-30 ml cell lysis buffer (25 mM TEA.HCl, pH 7.5, 0.15 M sodium chloride, 0.25 M sucrose, 2 mM EDTA, 2 mM EGTA, 0.25 mM DTT) and homogenized in a hand homogenizer to disrupt clumps of cells. The cell suspension was then sonicated on ice using a Heat Systems XL2020 Ultrasonic Processor. (Six 15 s pulses at power level 6 were usually sufficient to disrupt the cell walls and to shear the chromosomal DNA). Following sonication, cell debris was removed by centrifugation at 80000g for 30 minutes.

The supernatant was then dialysed into the column buffer (25 mM TEA.HCl pH 7.5, 0.2 mM EDTA, 0.25 mM DTT, 0.1 mM PMSF). The dialysate was centrifuged at 100000g for one hour to remove any insoluble matter and fractionated by ammonium sulfate precipitation. The 40-60% fraction was resuspended in 2-3 ml of column buffer and dialysed overnight against this buffer before being applied to an anion exchange column (DEAE-Sepharose™ Fast Flow, Pharmacia) of bed volume 50 ml. The light chain was eluted with a 0 to 0.4 M sodium chloride gradient over 40 ml. Fractions (of size 3-4 ml) containing pure light chain (as judged by SDS-PAGE) were pooled and dialysed extensively into 0.1%(w/v) ammonium bicarbonate containing 0.005%(v/v) β-mercaptoethanol and then freeze dried. Freeze dried powders were stored at -20°C until required.

Hybridization of wild type and mutant light chains into S1. The wild type and mutant light chains were combined with rabbit skeletal heavy chain using the ammonium chloride dissociation method (Wagner & Weeds, 1977) as modified in (Ueno & Morita, 1984). The isoenzymes were separated by ion exchange chromatography on SP-trisacryl M™ (Trayer & Trayer, 1988). The wild type HmAtELC was hybridized into S1(A2) whereas the HmAtELCΔ(1-45) and HmAtELCΔ(1-11) mutants were hybridized into rabbit skeletal S1(A1).

Fractions containing pure hybrid (as judged by SDS-PAGE) were pooled and concentrated by precipitation in 70%(w/v) ammonium sulfate. The precipitate was collected by centrifugation at 75000g for 15 minutes, resuspended in 1 ml buffer containing 5 mM TEA.HCl, pH 7.5, 0.25 mM DTT and dialysed into this buffer overnight. In order that control experiments with rabbit skeletal S1(A1) and S1(A2) matched as closely as possible the experiments on hybrid S1's, material for the controls was obtained by pooling the residual S1(A1) or S1(A2) in hybridization experiments and treating it as described above for the hybrids.

Actin activated MgATPase assays. Actin activated MgATPase assays were carried out by the discontinuous assay procedure (Winstanley et al. 1979), keeping the ionic strength low in order to magnify the difference between S1(A1) and S1(A2)-like kinetics. All assays were carried out in buffer containing 5 mM TEA.HCl, pH 7.5 at 25°C.

Actin was dissolved in buffer A (5 mM TEA.HCl pH 7.5, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.25 mM DTT) and dialysed vigorously overnight into this buffer. Insoluble material was pelleted by centrifugation at 80000g for 30 minutes and the resulting G-actin solution polymerized to F-actin by the addition of magnesium chloride to a final concentration of 2 mM. To keep the ionic strength constant following the addition of F-actin in buffer to the assay mix, compensating amounts of buffer A, plus 2 mM magnesium chloride, was added such that the final volume of buffer A was always 40 µl per ml of assay mix. S1 concentration varied between 0.10 and 0.16 µM.

Reactions were initiated by the addition of MgATP such that the final Mg^{2+} concentration was 2.5 mM and the final ATP concentration was 2.0 mM. Care was taken to ensure that no more than 30% of the ATP was consumed in each reaction. For each set of reactions a control was set up with no actin to correct for the non-actin activated MgATPase of S1 and any residual phosphate. Reaction rates were calculated from the amount of inorganic phosphate released during the course of the reaction (determined spectrophotometrically (Fiske & Subbarow, 1925), the zero actin control being used as a blank). Activities were calculated by dividing the rates (in μmol phosphate released s^{-1}) by S1 concentration. Data were analysed by non-linear curve fitting to the equation Activity = $(k_{cat}[Actin])/(K_M +[Actin])$ using the package Fig-P for Windows (Biosoft, Cambridge, UK). All points were weighted equally.

Cross-Linking. The procedure used for cross-linking was modified after Andreev & Borejdo, 1995. Freeze dried light chains were reconstituted in 5 mM TEA.HCl pH 7.5, 0.25 mM DTT in the presence of 1 mg DTT per mg freeze dried powder and 10 μl of 1 M sodium hydroxide per ml buffer, and dialysed overnight against this buffer. Actin was reconstituted as described above. The proteins were mixed in different molar ratios (see results) such that the total volume was 50 μl, and incubated at room temperature for one hour. After this time, EDC was added to a final concentration of 67 mM (ie 10 μl of 0.4 M stock giving a total volume of 60 μl), the reaction mixed gently and was allowed to proceed for a further hour. At this point the reaction was stopped by making the total volume into an SDS-PAGE sample and the products separated by SDS-PAGE.

Preparation of G-actin:DNase-I. Bovine pancreatic DNase I was reconstituted in 5 mM TEA.HCl, pH 7.5, 0.1 mM $CaCl_2$, 0.1 mM PMSF and dialysed against this buffer overnight. The solution was spun briefly in a bench top centrifuge to remove any insoluble matter, mixed with G-actin solution (prepared as described above) such that DNase I was in a 1.2-fold molar excess over actin and left at room temperature for 1 hour.

Analytical Methods. 12% SDS-PAGE (Weeds et al. 1975) gels (made up in 0.1 M Tris-Bicine buffer, pH 8.3) were run in 0.1 M Tris-Bicine, pH 8.3, 0.1%(w/v) SDS at

between 35 and 50 mA (constant current) and the products visualized by staining with Coomassie blue. Stacking gels were run with the separating gels as above and a stacking gel (about one-tenth of the length of the separating gel) composed of 3% polyacrylamide, made up in 0.3 M Tris.HCl, pH 6.9.

N-terminal amino acid sequencing (on an Applied Biosystems 473A automated protein sequencer) and automated DNA sequencing (on an Applied Biosystems 373A DNA sequencer) was carried out by AltaBioscience, the University of Birmingham. Matrix assisted laser desorption mass spectrometry (Hillenkamp & Karas, 1990) was carried out by Mr P Ashton, School of Chemistry, the University of Birmingham.

Protein concentrations were estimated by absorbance measurements using: $A_{280,1mg/ml}$(ELC) = 0.22 mg^{-1}.ml; $A_{280,1mg/ml}$(S1) = 0.80 mg^{-1}.ml; $A_{280,1mg/ml}$(DNase I) = 1.1 mg^{-1}.ml and $A_{290,1mg/ml}$(G-actin) = 0.63 mg^{-1}.ml with molecular masses as follows: wild type HmAtELC, 21 kDa; HmAtELCΔ(1-11), 20 kDa; HmAtELCΔ(1-45), 17 kDa; S1(A1), 112 kDa; S1(A2), 106 kDa; DNase-I, 31 kDa and actin, 42 kDa. The concentration of fluorescently labelled proteins was determined using the microtannin turbidity assay (Mejbaum-Katzenellenbogen & Dobryszycka, 1959).

RESULTS

Expression and Purification of Wild Type and Mutant Proteins. All three proteins expressed successfully. Expression was detected by the appearance of an additional band on SDS-PAGE of cell extracts before and after induction (Figure 2a). Levels of expression are high; we estimate that between 30 and 50% of total cell protein is HmAtELC or its mutants. Following sonication and removal of the cell debris by centrifugation, SDS-PAGE analysis showed the protein to be in the cytoplasm suggesting it is soluble in the bacterial cytoplasm and not localized to inclusion bodies.

Ammonium sulfate fractionation was used as a preliminary separation procedure. Only the 40-60% fractions contained any significant amount of material (Figure 2b). After dialysis to remove the ammonium sulfate these fractions were purified as indicated above. This stage of the purification procedure removed much of the nucleic acid contamination (as judged by the A_{260}/A_{280} ratio). The anion exchange column removed most of the remaining *E. coli* proteins. The wild type and mutant proteins behaved similarly on the column being eluted at approximately 0.25 M sodium chloride. Typical yields were between 70 and 150 mg protein per litre of bacterial culture. The purity and molecular mass of each protein was verified by matrix assisted laser desorption mass spectrometry (Hillenkamp & Karas, 1990). All three gave a single peak of approximately correct molecular mass (Table 1) and the start site of each protein was confirmed by N-terminal amino acid sequencing.

Hybridization of Recombinant Light Chains into Myosin Sub-Fragment 1. All three expressed ELCs could be recombined with S1 from rabbit skeletal muscle. The wild type ELC was hybridized into rabbit skeletal muscle S1(A2) and the hybrid was readily purified by ion-exchange chromatography on SP-Trisacryl M™ (Figure 3) eluting as a separate peak from the residual S1(A2) and free light chains. The HmAtELCΔ(1-45) and HmAtELCΔ(1-11) mutants were hybridized into S1(A1). With the HmAtELCΔ(1-45) mutant, the hybrid again eluted as a separate peak. However the S1(ELCΔ(1-11)) hybrid and residual S1(A1) eluted as a single peak, but SDS-PAGE analysis showed that the later fractions of this peak contained S1(A1) whereas the earlier ones contained pure mutant hybrid.

Actin Activated MgATPase Activities: All hybrids were biologically active: they all hydrolysed MgATP in an actin dependent manner. However, when the dependence of this activity on F-actin concentration was quantified, the two mutants fell into two clear groups. The wild type HmAtELC hybrid showed kinetics similar to rabbit skeletal S1(A1), whereas hybrids with both deletion mutants behave like S1(A2). These data are summarized in Table 2 and the plot for the wild type and mutant human atrial hybrids is shown in Figure 4.

Cross-Linking of wild type and mutant proteins to F-actin. Addition of the zero-length cross-linker EDC to a mixture of wild type HmAtELC and F-actin produces two additional bands on SDS-PAGE analysis: one at 63 kDa and one at 85 kDa (as compared to molecular mass markers, Figure 5a). The predicted size of the ELC-actin product is 63 kDa and, furthermore, this band does not appear when actin alone or ELC alone were subjected to the cross-linking reaction. Similar results are obtained using rabbit skeletal A1. Thus we conclude that this band results from a one to one complex of ELC and actin. With actin alone the 85 kDa band was observed and we assign this as an actin dimer. This conclusion is confirmed using fluorescently labelled actin and rabbit skeletal A1 (Figure 6). The 63 kDa band is labelled when either fluorescent rabbit skeletal A1 or fluorescent actin are used with unlabelled partners, but does not appear when the labelled proteins alone are exposed to the cross-linker. The 85 kDa band appears when actin alone is used. When the cross-linking experiment was repeated using the N-terminal deletion mutants, the 85 kDa band appeared, but no bands corresponding to a light chain-actin product was observed (Figure 5b). The results with free light chains are mirrored by their hybrids. The wild type hybrid gives the 63 kDa product on cross-linking to F-actin. The corresponding product with S1(A1) has been assigned as a light chain-actin adduct by several workers (Sutoh, 1982; Labbé et al. 1986; Andreev & Borejdo, 1995). The HmAtELCΔ(1-11) and HmAtELCΔ(1-45) hybrids do not produce this band on cross-linking (not shown). Thus we conclude that neither of the deletion mutants can be cross-linked to F-actin by this protocol.

With the wild type HmAtELC, the reaction gives greatest yield at a 1:1 molar ratio (Figure 5a) and is not particularly sensitive to ionic strength in the range 0 to 150 mM added sodium chloride (data not shown). The 65 kDa band was electroblotted onto a polyvinylidene fluoride membrane (Millipore) and N-terminal protein sequencing attempted. No sequence was obtained, suggesting that the N-terminal amino group is covalently linked to actin (there should be no interfering sequence from actin as this protein is N-terminally blocked (Collins & Elzinga, 1975)). However as rabbit skeletal fast muscle A1 light chain can also be cross-linked to F-actin (Figure 5b) and this protein is N-terminally blocked with trimethylalanine (Henry et al. 1982) (which cannot

take part in this cross-linking reaction), this result suggests that other residues must also be involved.

Cross-linking to G- and F-actin. The main interactions between F-actin and S1 involve the heavy chain at sites far from the ELC (Rayment et al. 1993a; Eldin et al. 1994). This raises the question as to whether the ELC binds to the same actin monomer as the main heavy chain sites, or to a separate one. That the cross-linking of A1 in S1(A1) is abolished at (non-physiological) 1:1 molar ratios (Andreev & Borejdo, 1995) suggests that it binds a second actin. The problem was resolved by comparing the cross-linking of A1-type ELCs, either "free" or in complex with the heavy chain to G- and F-actin. Since the heavy chain binds much more strongly to actin than the light chain, it would be expected that ELC-G-actin cross-linking would only be seen if the light chain interacts with same actin monomer as the heavy chain. This is shown schematically in Figure 7.

In order to be certain that the actin was monomeric, we stabilized G-actin by the addition of DNase-I. This forms a complex of extremely high stability ($K_d < 10^{-9}$ M (Mannherz et al. 1980)) and effectively traps actin in the monomeric state. The ability of G-actin-DNase-I to form a ternary complex with S1 has been established by others (Blanchoin et al. 1995) and in this laboratory (HR Trayer, personal communication). Using G-actin:DNase I we were able to show that with rabbit skeletal S1(A1), cross-linking only occurs between the heavy chain and actin: no actin-light chain cross-linked products were seen. This is not because G-actin:DNase I cannot interact with A1-type ELCs, since the free light chain can be cross-linked (Figure 8). The most likely explanation is that S1(A1) binds across two actin monomers with the stronger heavy chain sites interacting with one monomer and the ELC (and possibly some weaker heavy chain sites) with the next monomer along the filament (as depicted in the scheme in Figure 7).

DISCUSSION.

The human atrial myosin essential light chain can be readily expressed in *E. coli* and purified in high yield. This not only gives us the possibility of preparing (for the first time) an ELC which has never been exposed to denaturant during its purification (purification of A1 and A2 from rabbit skeletal muscle requires the use of urea to dissociate the light chain from the heavy chain) but also enables us to take a protein engineering approach to understanding the system. The kinetic assays clearly differentiate between the two classes of light chain - A1- and A2-like. HmAtELCΔ(1-11) falls into the A2-like class - demonstrating that the eleven N-terminal residues of the wild type light chain are required for modulation of the myosin motor. In contrast to the wild type, this mutant (and the A2-like, HmAtELCΔ(1-45)) does not interact with F-actin as shown by chemical cross-linking. Thus we are able to locate the actin binding site to within these eleven N-terminal residues and establish a correlation between actin binding and kinetic modulation.

A recent cross-linking study (Andreev & Borejdo, 1995) suggests that S1 binds across two actin molecules in the filament, with the main sites of heavy chain-actin interaction on one monomer ("actin 1" in Figure 7) and a secondary heavy chain interaction and the ELC-actin interaction being on actin 2. We were able to confirm this directly by comparing the cross-linking of S1(HmAtELC) and free HmAtELC to F-actin and G-actin:DNAase I. The free light chain binds to both but HmAtELC in S1(HmAtELC) binds to F-actin only. This result explains why rabbit fast skeletal muscle S1(A1) and S1(A2) bind to G-actin with equal affinity (Blanchoin et al. 1995) whereas they can be separated by F-actin affinity chromatography (Winstanley et al. 1977).

We assume that our success in cross-linking the free ELC to actin results from our use of a simpler (and more direct) protocol which allows the molecules to come to equilibrium and then introduces the cross-linker. Cross-linking is a powerful tool for investigating relatively weak interactions (which we assume the ELC-actin interaction is) as it captures molecules in the bound state even if this bound state is rare.

Although we have demonstrated that an actin binding site in the first eleven residues of A1-type ELCs fine tunes the activity of the myosin motor, many questions remain to be

answered. Perhaps the most obvious is how can the light chain and actin come into contact when a large (>8 nm) gap is apparent from the models of acto-S1 derived from the crystal structures of the two proteins (Rayment et al. 1993a). However, none of the residues in the N-terminal extension of A1 are resolved in this structure (probably due in part to the high segmental mobility of this region in the absence of F-actin and the use of a mixture of S1 isoenzymes in the crystallization process). Furthermore, this is a model of acto-S1 and at best represents only one of many conformations of the two proteins. In addition, in electron microscopy image reconstructions of acto-S1 additional electron density close to the C-terminus of actin is seen when S1(A1) is used that is not present when S1(A2) used (Milligan et al. 1990); a result which has recently been repeated (Waller et al. 1995).

An explanation for the apparent contradiction between these two methods of visualising acto-S1 may come from considering the remaining thirty amino acid residues of the N-terminal extension on A1-type ELCs which is rich in (Xxx-Pro) repeats (Lys.Pro(Ala.Pro)$_8$ in HmAtELC). NMR studies on this system (Bhandari et al. 1986) and others (Brewer et al. 1990), molecular modelling (Abillon et al. 1990) and x-ray crystallographic analysis of model peptides (Di Blasio et al. 1992) show that such sequences adopt extended, rod-like structures. Such a structure might be crucial in spanning the gap between the ELC and the actin filament and thus in correctly positioning the actin binding site. This has the additional advantage that placing the binding site on the end of an inflexible rod reduces the problem of locating the site on actin from one of three-dimensional diffusion to one of two-dimensional diffusion over a limited area - effectively increasing the affinity of the interaction. It should also be noted that the distance to be bridged between the ELC and either actin 1 or actin 2 in the scheme in Figure 7 is approximately the same in models of the acto-S1 complex (Rayment et al. 1993a).

Although we have narrowed down the site of interaction to within eleven residues, we can say nothing about the role of individual amino acids. NMR studies on the intact light chain (Henry et al. 1985a; Henry et al. 1985b; Trayer et al. 1987) implicate the N-terminal alanine and lysine residues but can say nothing about which lysines. This

agrees with our observation that we were unable to sequence the actin-ELC cross-linked product. Site directed mutagenesis studies are currently underway to probe the rôle of individual amino acids in both kinetic modulation and actin binding.

However, the greatest unanswered question is how information is transmitted from the ELC-actin interface to the S1 active site some 8 nm away. The model in Figure 7 suggests two possible pathways: one in which information passes through S1 and an alternative pathway in which it passes through the actin filament. Access to recombinant A1-type light chain, an assay system which differentiates between the two kinetic types and a method for monitoring the direct light chain-actin interaction provide the means for answering these questions.

In conclusion, our results are consistent with physiological studies which suggest that the ELC, in striated muscle, fine tunes the myosin motor, making possible subtle changes in the contractile properties of muscle fibres (Sweeney et al. 1988; Bottinelli et al. 1994a; Schiaffino & Reggiani, 1994).

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Table 1. Molecular masses of expressed proteins as determined by matrix assisted laser desorption mass specrometry[a]

Protein	**Calculated Molecular Mass[b] / Da**	**Observed Molecular Mass / Da**	**Difference (%)**
Wild type HmAtELC	21564	21359	-0.95
HmAtELCΔ(1-45)	17080	17088	+0.05
HmAtELCΔ(1-11)	20360	20238	-0.60

[a] Determined as described in Experimental Procedures.

[b] The wild type protein mass was determined from Arnold et al. 1988.

Table 2. Kinetic constants for MgATP hydrolysis of native and hybrid S1s[a].

Hybrid / Native Protein[b]	Apparent K_M for F-actin / μM [c]	k_{cat} / s^{-1} [c]	n [d]	Phenotype
S1(HmAtELC)	5.4±1.5	15.3±2.8	32	A1-like
S1(HmAtELCΔ(1-11))	20.8±6.9	27.3±7.7	34	A2-like
S1(HmAtELCΔ(1-45))	38.9±16.7	43.0±16.8	22	A2-like
Rabbit skeletal S1(A1)	4.2±0.6	13.7±1.1	20	A1-like

Rabbit Skeletal S1(A2)	31.9±9.9	34.6±9.5	24	A2-like

[a] The methods for measuring the MgATPase activitites and analysing the results are given in the experimental procedures.

[b] The rabbit skeletal S1s were prepared from fast muscle as described in Experimental Procedures. The HmAtELC and its mutants were expressed in *E. coli* and hybridized to native rabbit skeletal S1 heavy chains as described in Experimental Procedures.

[c] Values are given ± standard error.

[d] The number of determinations.

FIGURE LEGENDS

Figure 1. Alignment of the first 99 amino acids of various ELCs. The numbering is based on the human atrial ELC sequence, not counting the initial methionine. Note that the cloning process introduces a Met residue at the N-terminus of the mutants. The sequences were obtained from Frank & Weeds, 1974 (rabbit skeletal proteins) and Arnold et al. 1988 (human atrial isoform). RbSkA1, rabbit skeletal fast muscle A1-type ELC; RbSkA2, rabbit skeletal fast muscle A2-type ELC; other abbreviations given earlier.

Figure 2. Expression and purification of wild type HmAtELC. (A) SDS-PAGE of *E. coli* cell lysates before (lane 1) and two hours after induction with IPTG (lane 2). (B) Ammonium sulfate fractionation of *E. coli* cell lysates. Lane 1, total cell extract; lane 2 0-30%(w/v) ammonium sulfate precipitate; lane 3 30-40%(w/v) ammonium sulfate precipitate; lane 4 40-50%(w/v) ammonium sulfate precipitate; lane 5 50-60%(w/v) ammonium sulfate precipitate; lane 6 60-70%(w/v) ammonium sulfate precipitate; lane 7 70-80%(w/v) ammonium sulfate precipitate and lane 8 80% (w/v) supernatant. (C) Elution from DEAE-Sepharose Fast Flow column (50 ml) in 25 mM TEA.HCl, pH 7.5, 2.5 mM EDTA, 0.25 mM DTT, 0.1 mM PMSF. At the arrow, a gradient of 0 to 0.4 M sodium chloride over 500 ml was applied and 3 ml fractions collected. The area indicated by the bar was pooled and used in subsequent experiments. Solid squares,

elution of proteins measured by absorbence at 280 nm; solid circles, sodium chloride concentration. The insert shows SDS-PAGE of the 40-60% ammonium sulfate fractions applied to the column (lane 1) and the pooled fractions (lane 2).

Figure 3. Separation of S1 isoforms on SP-Tris acryl after hybridization. (A) After hybridzation of the HmAtELC into rabbit skeletal S1(A2) (see experimental procedures) the sample was applied to an SP-Tris Acryl column (50 ml) in 10 mM MOPS buffer, pH 7.5. At the arrow a gradient from 0 to 0.2 M sodium chloride over 200 ml was applied. The bars mark where pools 1 and 2 were collected. Solid squares, elution of proteins measured by absorbence at 280 nm; solid circles, conductivity. (B) SDS-PAGE of the resolved S1 molecules. Lane 1 applied hybridisation sample; lane 2 pool 1; lane 3 pool 2. Lanes 4 and 5 show the hybrids S1(HmAtELCΔ(1-11)) and S1(HmAtELCΔ(1-45)) respectively purified exactly as in (A).

Figure 4. A plot of MgATPase activity against F-actin concentration for the various mutant ELC S1 hybrids. Solid circles, S1(HmAtELC); solid squares, S1(HmAtELCΔ(1-11)); solid triangles S1(HmAtELCΔ(1-45)). Kinetic constants were determined by non-linear curve fitting to the equation: Activity = $(k_{cat}.[actin])/(K_M + [actin])$.

Figure 5. Cross-linking of the wild type and mutant ELCs to F-actin. The cross-linking was carried out with EDC as described in the Experimental Procedures. (A) Cross-linking of wild type HmAtELC to F-actin. Lanes 1 to 6 contain cross-linking experiments carried out at molar ratios of F-actin:HmAtELC of 2:1, 1:1, 1:2, 1:4, 1:8 and 1:16; lanes 7 and 8 HmAtELC and F-actin respectively after cross-linking with EDC. Protein concentrations were: F-actin 20 μM in lanes 1,2,3 and 8; 10 μM in lane 4; 5 μM in lane 5; 2.5 μM in lane 6 and HmAtELC 40 μM in lanes 3,4,5,6 and 7; 20 μM in lane 2 and 10 μM in lane 1. The identification of the actin-ELC and actin-actin adducts was made after comparison with molecular mass markers (lane 9), Figure 6 and

with cross-linked actin alone (lane 7). (B) Cross-linking of rabbit skeletal A1-type ELC, HmAtELCΔ(1-45) and HmAtELCΔ(1-11) to F-actin. Lanes 1-3 actin:rabbit skeletal A1 ratios of 2:1, 1:1 and 1:2; lane 4 rabbit skeletal A1 alone; lanes 5-7 actin:HmAtELCΔ(1-45) ratios of 2:1, 1:1 and 1:2; lane 8 HmAtELCΔ(1-45) alone; lanes 9 to 11 actin: HmAtELCΔ(1-11) ratios of 2:1,1:1 and 1:2; lane 12 HmAtELCΔ(1-11) alone. Proteins concentrations were: actin 20 μM in all lanes containing actin; light chain concentrations varied between 10 μM and 40 μM. The presence of the actin dimer but the absence of the mutant ELC-actin adducts in lanes 5 to 7 and 9 to 11 should be noted. It is our experience that the various ELCs do not run as sharp bands after the cross-linking reaction.

Figure 6. Cross-linking of F-actin and HmAtELC labelled with 1,5-IAEDANS and visualised under ultraviolet light. Lane 1, IAEDANS-F-actin and unlabelled HmAtELC; lane 2, unlabelled F-actin and IAEDANS-rabbit skeletal A1-type ELC; lane 3, IAEDANS-rabbit skeletal A1-type ELC; lane 4, IAEDANS-F-actin. The proteins were labelled with 1,5-IAEDANS as indicated and all four samples were treated with EDC as described in Experimental Procedures. The actin dimer (and higher molecular mass adducts) are clearly seen in lanes 1 and 4 and the actin-ELC adduct in lanes 1 and 2.

Figure 7. Schematic diagram of the possible modes of cross-linking of S1 with an A1-type ELC to F-actin and G-actin-DNase-I.

Figure 8. Cross-linking of wild type human atrial ELC and rabbit skeletal S1(A1) to G-actin-DNase-I. Lane 1, 10 μM rabbit skeletal S1(A1); lane 2, 10 μM S1(A1) plus 10 μM G-actin-DNase-I; lane 3 10 μM S1(A1) plus 20 μM G-actin-DNase-I; lane 4, 10 μM HmAtELC plus 5 μM G-actin-DNase-I; lane 5, 10 μM HmAtELC plus 10 μM G-actin-DNase-I; lane 6, 10 μM HmAtELC plus 20 μM G-actin-DNase-I; lane 7, 10 μM HmAtELC. In all cases the cross-linker EDC was present in the reaction mixture. Note that the 63 kDa band corresponding to the ELC-actin adduct is seen only with the free

light chain and not with S1(A1). The higher molecular mass adducts can be attributed to actin-DNase and myosin heavy chain-actin products. HC, myosin heavy chain.

3.2B ADDITIONAL INFORMATION.

3.2B.1 Development of the Purification Strategy for the Recombinant Human Atrial ELC.

Initial attempts to purify the wild type, recombinant human atrial ELC were based on established procedures for separating this protein from rabbit skeletal muscle extracts (Trayer and Trayer, 1988). The protein has a low isoelectric pH (about 4.2) and is therefore negatively charged at near neutral pH and interacts with positively charged resins. The soluble proteins extracted from *E. coli* expressing the light chain were applied to a DEAE-Sepharose column and eluted with a 0 to 0.4 M sodium chloride gradient. This procedure produced fractions which were enriched in HmAtELC, however these fractions were contaminated with nucleic acid (as judged by absorbance scans in the range 230 to 300 nm). The nucleic acids interacted strongly with this column and this interfered with the binding properties of the protein causing it to smear out over many fractions. The fractions containing ELC were pooled and concentrated by extensive dialysis against 0.1 %(w/v) ammonium hydrogencarbonate, 0.05 %(v/v) β-mercaptoethanol, freeze drying and reconstituting in a smaller volume of column buffer before being applied to a second positively charged resin (DEAE-Sepharose Fast Flow, DEAE-FF) from which the ELC was eluted with a 0 to 0.4 M sodium chloride gradient. Fractions containing almost pure HmAtELC (as judged by Coomassie Blue stained SDS-PAGE gels) were obtained which contained very little contaminating nucleic acid. However, the yield was relatively low at about 18 mg purified protein per 1 L bacterial culture.

Given that the large quantity of nucleic acid in the bacterial cell extract appeared to be the principal problem, the method was re-designed to remove nucleic acids in the first stage and to purify the ELC with respect to other proteins in the second. The cell extract was applied to a DEAE-FF column and (after washing to remove unbound material) the majority of the bound proteins eluted with a 0.4 M sodium chloride step. The protein containing fractions (which contained relatively small amounts of nucleic acid) were pooled, dialysed back into the column buffer and re-applied to a second

DEAE-FF column and eluted with a 0 to 0.4 M sodium chloride gradient. The material produced by this method was less pure than that produced by the original method, but the yield was much improved (typically 70 to 100 mg protein per 1 L bacterial culture). If material of a higher quality was required, the majority of protein contaminants could be removed by passage down a gel filtration (Sephadex G100) column (work carried out by Miss Sue Brewer).

The method was further improved by replacing the first DEAE-FF column with ammonium sulphate fractionation as described in Paper I. This method resulted in material of similar purity as the two column method, but incorporated considerable savings in time.

3.2B.2 Anomalous Expression of the HmAtELC.Δ(1-45) (A2-like) Mutant.

Protein expression was routinely monitored by the appearance of an additional band on SDS-PAGE analysis (see section 2B.7 and Paper I). However, when the expression of the HmAtELC.Δ(1-45) mutant was assessed in this manner, two bands were seen (figure 3.1,I.)

The lower band has a similar mobility to rabbit skeletal A2 (figure 3.1,II) suggesting that this is the band corresponding to the HmAtELC.Δ(1-45) mutant rather than the upper band with the lower one being a breakdown product. Both bands were transferred onto PVDF membrane and subjected to N-terminal sequencing and the same sequence (MDFTA, which corresponds to the expected amino acids from the N-terminus of the HmAtELC.Δ(1-45) mutant) was obtained in both cases. Both proteins therefore result from expression of the mutant and any differences between the two are not at the N-terminus. DNA sequencing showed the correct sequence throughout and the presence of a stop codon at the 3′ end. The high molecular mass band could either result from a misfolded product which runs anomalously on SDS-PAGE gels or from a product which has additional amino acids (presumably at the C-terminus, which would require translation to “run though” the stop codon).

To address this problem, the mutant was expressed in a medium scale (50 ml) culture and the cells collected by centrifugation. The cell pellet was taken up in 25 mM TEA.HCl, pH 7.5, 2.5 mM EDTA, 0.5 mM DTT, 6 M urea and sonicated. The presence of urea in this buffer should have unfolded all the proteins in the cell extract. SDS-PAGE analysis of the soluble material after sonication showed the presence of both bands. The insoluble matter contained only the upper band (figure 3.1,III). This experiment argues in favour of there being two species of different molecular masses rather than two differently folded forms of the same protein.

Preparation of this mutant according to the standard protocol (section 2C.2.3 and Paper I) in the absence of urea resulted in two fractions after sonication: the soluble fraction contained the lower band protein (which could be purified further according to the standard method) and insoluble fraction contained the upper band (figure 3.1,IV). Furthermore, the lower band protein once purified, gave a molecular mass (by MALDI-MS) of 17088 Da as compared to the predicted value of 17080 Da confirming that it was the protein of interest.

The nature of the upper band protein remains obscure. T7 RNA polymerase is known to "read through" the termination sequence (up to one in ten times) and produce longer mRNA transcripts (Carter *et al.* 1981). However, there is no report of such transcripts resulting in "read through" translation. Even if this is occurring in our case, the question remains as to why it only occurs in this mutant which is after all only a shorter version of the wild type and therefore contains no new "signals" to trigger such an event. That the unwanted higher molecular mass protein is in the insoluble fraction after sonication in non-denaturing buffer, suggests that it is localised to inclusion bodies (or some other membrane bound structure). Furthermore, it was observed that expression for relatively long periods of time (about 5 hours) increased the ratio of lower to upper bands on SDS-PAGE (not shown) suggesting that the higher molecular mass protein is less stable. Since the two proteins could be separated at the sonication stage, this anomalous expression presented no further problems to the development of the project.

3.2B.3 Cross-Linking of N-terminal peptides to F-actin.

Further evidence for the existence of an actin binding site in the N-terminal extension of A1-type ELCs is provided by the successful cross-linking of a peptide corresponding to residues 1-32 of rabbit skeletal A1 (figure 3.2). The protocol used was essentially identical to that used for cross-linking whole ELCs to F-actin (section 2C.7.1 and Paper I).

3.2B.4 Cross-Linking: Effect of Ionic Strength.

The effect of ionic strength on the cross-linking reaction between HmAtELC and F-actin was investigated by supplementing the standard cross-linking reaction (section 2C.7.1 and Paper I) with increasing concentrations of added sodium chloride. As can be seen from figure 3.3, the extent of cross-linking does decrease slightly with increasing ionic strength, but even at 150 mM added sodium chloride, the cross-linked product is still clearly visible. This contrasts with reports that increasing salt concentrations disrupt A1-actin cross-linking in experiments using S1(A1) (Yamamoto and Sekine, 1983). This may be due to differences in the protocol used (these authors added the cross-linker immediately after mixing the proteins and did not pre-incubate them to allow them to come to equilibrium) or due to effects of ionic strength on the heavy chain-actin interactions.

3.2B.4 Cross-Linking: Effect of Troponin I.

Potentially, cross-linking could be used to investigate the rôle (if any) of TnI in the modulation of myosin motor kinetics by A1-type light chains. Specifically it could answer the questions of how the binding of TnI to actin affects the interaction of A1 with actin and whether a ternary complex (A1-actin-TnI) can be formed. However, before this can be done it is necessary to find conditions under which TnI can be reproducibly cross-linked to F-actin.

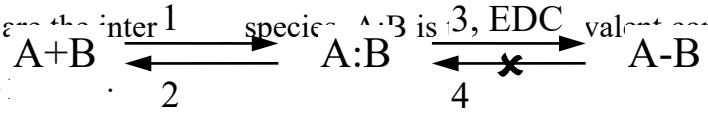
Human cardiac TnI was mixed with F-actin in various molar ratios (ranging from TnI:actin = 1:4 to 4:1) and in each case a band of approximately the correct molecular mass (65 kDa) for an actin-TnI product was seen on cross-linking (figure 3.4). However, a band of similar size (although lower intensity) was seen when TnI alone was exposed to the cross-linker. This suggests that this band results at least in part from the formation of TnI trimers. Even if actin-TnI products are also produced, the formation of this trimer would make any subsequent interpretation difficult.

Addition of TnI to mixtures of HmAtELC and F-actin (ELC:actin:TnI = 1:1:1) does not affect the cross-linking of the ELC to actin (not shown), nor are any bands corresponding to an ELC-actin-TnI complex seen. However, given that TnI may not even cross-link to actin under these conditions it is difficult to draw any conclusions from these experiments.

3.3 Quantitative Estimation of the ELC-Actin Interaction.

3.3.1 Why Cross-Linking is Inappropriate.

The development of a reproducible method of cross-linking HmAtELC and F-actin gave a rapid and reliable method for assessing whether, or not, a particular mutant binds to actin. However it is not a good method to quantify the interaction. Cross-linking does not report on the equilibrium situation (unlike, say, fluorescence) but captures complexes once formed and prevents the reverse reaction:



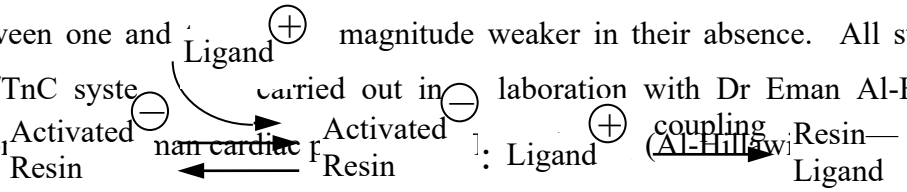
where A and B are the interacting species, A:B is the non-covalent complex and A-B is the cross-linked product.

The amount of A-B is controlled by a number of factors other than the equilibrium of A + B and A:B (ie reactions 1 and 2 in the above scheme) - especially the rate of reaction 3 which will be controlled by the EDC concentration (and hence the rate of EDC breakdown, which would be very difficult to quantify) and the availability of suitable reactive groups in A and B. So, although under conditions that are otherwise equal, cross-linking can be used to compare, qualitatively, the binding of similar proteins (see for example sections 3.2B.5 and Paper III) other methods are more appropriate for determining binding constants.

3.3.2 Surface Plasmon Resonance.

3.3.2.1 System Evaluation with Cardiac TnI and TnC.

The calcium-sensitive TnC/TnI interaction was chosen as a good model system as the interaction has been well characterised *in vitro*. The interaction is strong in the presence of calcium ions ($K_d \sim 10^{-8}$ M (Al-Hillawi *et al.* 1995; Farah and Reinach, 1995)) and between one and [illegible] magnitude weaker in their absence. All studies on the TnI/TnC syste[illegible] carried out in [illegible]laboration with Dr Eman Al-Hillawi using recom[illegible]nan cardiac p[illegible]



Initial attempts to immobilise cd TnC (0.5 $mg.ml^{-1}$ in 10 mM acetate buffer, pH 4.8, running buffer 10 mM MOPS pH 7.3 with NaOH, 0.5 mM DTT, 150 mM sodium chloride) proved unsuccessful (figure 3.5a). The highly negatively charged nature of the carboxymethyl dextran (CM dextran) matrix on the surface of the chip means that proteins carrying a net positive charge (ie below their isoelectric points) will be attracted to the surface - an event which is a pre-requisite for covalent attachment.

Unfortunately, the isoelectric point of cd TnC is estimated (using the GCG program PEPTIDESORT (Devereux *et al.* 1984)) to be 3.9 and the solubility of this protein is minimal below pH 4.5. It seemed likely that the failure to immobilise cd TnC resulted from the failure to form a TnC:activated resin complex at this pH. This was confirmed by passing cd TnC (in the same buffer as above) over an underivatised CM dextran surface. If there is any interaction with the surface, an increase in the signal should have been observed. No significant increase was seen.

$$TnC + TnI_{(imm)} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} TnC{:}TnI_{(imm)}$$

Attempts to reduce the effective electrostatic charge within the resin by including relatively high salt concentrations (0.5 M sodium chloride) in the running buffer (otherwise as before) did not improve the situation. Nor did derivatising the surface with ethylenediamine ($H_2N(CH_2)_2NH_2$) (O'Shannessy *et al.* 1992) or lysine ($H_2NCH((CH_2)_4NH_2)COOH$) in an attempt to introduce some positive charges onto the surface. Our preference would have been to immobilise cd TnC (since we have a range of TnI and TnT mutants available), but given the highly acidic nature of the only commercially available resin, this seems unlikely to be possible using the wild type protein (but see below).

TnI on the other hand, is a highly basic protein (isoelectric point for the human cardiac form is estimated at 11.5) and there is significant electrostatic interaction between this protein and the matrix at pH 7.3. Consequently, the immobilisation of cd TnI (running buffer as for TnC, 0.2 $mg.ml^{-1}$ dissolved in this buffer) proceeded well (figure 3.5b).

The CM-dextran surface derivatised with immobilised cd TnI was used for interaction analysis with cd TnC (running buffer as before, with the addition of 2 mM calcium chloride, stock solution of cd TnC dissolved in this buffer) (figure 3.5c). Binding clearly occurs with curves showing a saturatable rise when TnC is injected and an exponential decay when the surface is subsequently washed with buffer. The data were analysed by fitting to the model expected if the interaction obeys simple, second order association and first order dissociation kinetics, ie:

Where the sub-script (imm) indicates a component which is covalently coupled to the matrix. The extent of reaction is reported by the change in response, R and the dissociation phase described by the equation:

$$R = R_0.\exp(-k_d.(t-t_0)$$

where R_0 is the response (in RU) at time t_0, the time at the first point picked for fitting; t is the elapsed time and k_d is the first order dissociation rate constant as defined in the reaction scheme above. The association phase is described by:

$$R = ((R_{max}.C.k_a)/(k_a.C.n + k_d)).(1-\exp((-k_a.C.n + k_d)(t - t_i)))$$

where R_{max} is the maximum binding capacity of the surface (in RU), C is the concentration of analyte, n is the number of equivalent binding sites per molecule (1 in this case) and t_i is the actual injection time. Fitting to these equations is clearly flawed as shown by the magnitude and non-random nature of the residuals (figures 3.5d,e) and the dependence of the rate constant on concentration (figure 3.5f). There is no reason to suspect a more complex model for this system and binding data obtained by other methods has been analysed on the assumption of this model eg (Al-Hillawi *et al.* 1995).

The binding of soluble analyte to immobilised ligand in the flow-cell can be represented as a two stage process:

$$TnC_{(bulk)} \underset{k_{-M}}{\overset{k_M}{\rightleftharpoons}} TnC_{(resin)} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} TnC_{(resin)}.Tnl_{(imm)}$$

where the sub-script (bulk) indicates a component in the solution phase; the sub-script (resin) represents a component that has entered the CM dextran layer. Thus, the first equilibrium describes the entry of the analyte into the resin. In the absence of any interaction between the analyte and the resin, k_M and k_{-M} will be equal (ie the equilibrium constant for the process will be unity) and k_M will describe the diffusional mass transport of the analyte into the CM dextran layer. Since the aim of the experiment is to measure k_a and k_d, the reaction controlled by k_a should be made rate limiting (if possible). This is most readily achieved by lowering the amount of

immobilised ligand (typically to less than 500 RU) since the rate of this reaction is given by $k_a.[TnC_{(resin)}].[TnI_{(imm)}]$.

In our case, however, TnC (with net negative charge at pH 7.3) is repelled from the matrix and k_M will be lower than k_{-M} (ie the equilibrium favours $TnC_{(bulk)}$). This means that the first stage of the reaction sequence dominates the process. Although some 1300 RU TnI were immobilised (far higher than would be normally used for kinetic analysis), the R_{max} values derived from the data (approximately 150 RU, note that only the *rate* and not the *extent* of complex formation is controlled by the first stage of the reaction scheme) suggest that only about 10% of the immobilised protein is active (as judged by its ability to bind to TnC in the presence of calcium ions) a result that is not surprising given the difficulty in binding TnC to TnI immobilised on affinity columns (IP Trayer, personal communication). This suggests that reducing the amount of TnI immobilised would not improve the situation significantly since the amount of *active* TnI bound is already within acceptable limits. When the 2 mM calcium chloride in the buffer was substituted for 2 mM EGTA no significant differences in the binding properties were seen, showing that the experiment fails to report the differences in binding between the Ca^{2+}-bound and Ca^{2+}-free states.

Although cd TnI can be immobilised to the CM dextran surface, attempts to interact it with cd TnC appear to yield meaningless data, due at least in part to the charged nature of this surface.

The problem of immobilising cd TnC to the CM dextran surface can be over-come by expressing the protein with glutathione-S-transferase (GST) fused to the N-terminus (Stuart Downing, final year undergraduate research project) and capturing this fusion protein on a surface previously derivatised with a monoclonal antibody to GST (illustrated schematically in figure 3.6). This method results in stably bound GST-TnC (ie the k_d for the anti-$GST_{(imm)}$:GST-TnC complex is very low) (figure 3.5g,h). (Although, interestingly, a larger amount of GST alone bound to the same surface suggesting that electrostatic repulsion is still a factor). However, attempts to monitor the interaction of this fusion protein with cd TnI failed due to the high degree of non-

specific, electrostatic interaction between cd TnI and the CM dextran which masked any interaction (not shown). (The GST-TnC fusion was shown to be capable of binding to cd TnI in a calcium sensitive manner by alkaline urea gels (Stuart Downing, final year undergraduate research project)).

Another promising method for immobilising cd TnC is by use of a mutant in which both naturally occurring cysteine residues have been replaced with serine (Dr HR Trayer, unpublished) and a $(Gly)_3Cys$ tail added to the C-terminus (Sophie Milson, final year undergraduate research project). Attempts were made to couple this protein to the CM dextran surface using the surface thiol method (section 2D.1.3.2, (figure 3.5i)). While this initially appeared to result in stably bound material (indicated by an increase in the baseline) this could not all be removed by washing with 100 mM DTT, raising the possibility that the increase in response observed was due to non-covalent aggregation and precipitation of material on the surface.

This mutant could be biotinylated at the single, C-terminal cysteine (using MPB, section 2C.8.2.3) and coupled to CM dextran surfaces derivatised with streptavidin. Attempts to do so gave ambiguous results, which were ascribed to low yields of biotinylated product (Sophie Milson, final year undergraduate research project).

In conclusion, the highly negatively charged nature of the CM dextran resin make quantitative studies with highly charged proteins such as TnI and TnC very difficult, if not impossible. TnC is repelled from the matrix making direct immobilisation difficult and interfering with the kinetics when used as an analyte. TnI interacts so strongly with the resin that non-specific binding is likely to be at least an order of magnitude greater than any specific interaction making any analysis impractical. It will not be possible to carry out any quantitative analysis on this system until surfaces with lower (or preferably zero) surface charge are commercially available.

3.3.2.2 Immobilisation of G- and F-actin and the use of these proteins in interaction analysis.

Actin is less acidic than cd TnC (its isoelectric point is estimated as 5.2); however, reducing the pH to less than about 6.0 results in irreversible aggregation, precipitation and denaturation. Glutaraldehyde cross-linked F-actin (2.5 $mg.ml^{-1}$ in the dialysis buffer described in section 2C.7.2, running buffer the same) failed to immobilise (figure 3.7a), as did G-actin in buffer A (as section 2C.9 except MOPS buffer was used in place of TEA.HCl) (not shown). Again this was most likely due to repulsion of the negatively charged protein by the resin (*cf* TnC).

G-actin forms a binary complex of extremely high affinity with DNase-I (Mannherz *et al.* 1980) and so an attempt was made to immobilise G-actin *via* DNase-I (in a similar manner to the capturing of GST-TnC by Anti-GST). Although DNase-I has a low iso-electric point (estimated as 5.0) it can be kept in solution at reasonable concentrations at pH values below this. Bovine pancreatic DNase-I (approximately 5 $mg.ml^{-1}$ in acetate buffer pH 4.8 and running buffer as for the immobilisation of G-actin) was immobilised successfully (figure 3.7b). However, the injection of G-actin (0.5 $mg.ml^{-1}$ in buffer A) across this surface results in no change (not shown).

There are a number of potential explanations for this. DNase-I could be denatured at the low pH used for immobilisation or by the immobilisation itself, or it may be that repulsion by the CM-dextran matrix is still a significant factor.

Maleimido reagents label actin specifically at Cys373 and an attempt to biotinylate actin with MPB (section 2C.8.2.3) failed due (probably) to the low solubility of the reagent in buffer A and so we turned to G-actin biotinylated at surface lysine residues, which was available as a commercial product. This readily immobilised onto a CM dextran surface derivatised with streptavidin (0.5 $mg.ml^{-1}$ actin in buffer A, running buffer the same) (figure 3.7c). The amount immobilised (120 RU) would be ideal for kinetic analysis. However, the immobilised actin did not interact with HmAtELC (0.4 $mg.ml^{-1}$) (figure 3.7d), S1(A1) (1.6 $mg.ml^{-1}$) and DNase-I (1.2 $mg.ml^{-1}$). While it is possible that the binding constant for G-actin and the ELC is too low to be detected (k_a less than 10^{-4} $M^{-1}s^{-1}$) the results with S1 and, especially DNase-I are more worrying. The manufacturers of the biotinylated actin claim an average labelling of one biotin per actin, which, given

that there are several exposed lysine residues on the surface of actin, means that there will be heterogeneity of immobilised ligand. It seems unlikely that all forms are unable to interact with all three proteins (all of which interact in solution, see paper I). A more likely explanation is that the large number of acidic groups in the matrix are not fully buffered and that the prevailing pH in the CM dextran layer is lower than in the bulk phase. (The buffer concentration and the ionic strength had been kept low in order to favour G-actin over F- and because all previous reports of A1-type ELC actin interaction were at low ionic strength (Henry *et al.* 1985b; Trayer *et al.* 1987; Winstanley *et al.* 1977)). This low pH environment may cause the irreversible denaturation of immobilised actin. There is some evidence in favour of this explanation: it was observed that the baseline after immobilisation of biotin-G-actin was unstable, a feature which is characteristic of slow changes (eg denaturation and aggregation) occurring within the matrix.

Once again, it seems that until alternative surfaces become available it will be impossible to immobilise actin in an active form. Our preference would be to immobilise F-actin (probably stabilised by glutaraldehyde cross-linking) as studies on the non-physiological G-actin-DNase-I complex would be of limited value.

3.3.2.3 Future prospects for the use of the BIAcore in the investigation of this system.

Potentially, kinetic studies of the interaction between actin binding proteins and actin could reveal much valuable information. The binding constant for the A1-type ELC-actin interaction has never been determined. This method would also give the association and dissociation rate constants. This would enable us to assess the differences in binding to F-actin by our site-directed mutants (see Chapter 5) but also whether changes (if any) result from differences in k_a or k_d, or both. The results of Paper I could be confirmed by peptide competition experiments using peptides corresponding to the actin binding site (for a description of this methodology see (Karlsson, 1994)). Since the wild type human atrial ELC-actin interaction is thought to be a single site interaction, peptides which encode the entire binding site should interact

with the same affinity. In a similar fashion, the site of interaction on actin could be located.

The facility to control the temperature of the flow cell in the BIAcore 2000 makes it possible to determine the thermodynamic parameters of the interaction. ΔG can be determined directly from the binding constant K_A (since $\Delta G = -RT.\log_e K_A$). The temperature dependence of the equilibrium constant can be used to determine ΔH (assuming this parameter to be temperature independent in the first approximation) according to: $\log_e(K_{T1}/K_{T2}) = -(\Delta H/R).(T_2^{-1} - T_1^{-1})$, where K_{T1} and K_{T2} are the equilibrium constants at the absolute temperatures T_1 and T_2 respectively and R is the molar gas constant. Thence the entropy change at each temperature can be determined from: $(\Delta H - \Delta G)/T = \Delta S$ (Moore, 1972).

However, such studies will have to wait until alternative matrices and suitable immobilisation chemistries become available (see also section 7.3).

3.3.3 Future Prospects

The binding constants of many actin binding proteins have been determined by co-sedimentation experiments with F-actin (see eg (Way *et al.* 1992; Trayer and Trayer, 1985)). This method might be applicable to the free ELC (and could certainly be used to quantify any differences between hybrids with the various mutants), but only if the dissociation rate (k_d) is lower than the sedimentation rate, which may not be the case if the binding constant is low.

The site of interaction on actin is thought to be close to the C-terminus (Trayer *et al.* 1987; Milligan *et al.* 1990). Fluorescent labels can be placed on Cys 373 using maleimido or iodoacetamido derivatised labels (see eg (Trayer and Trayer, 1988)) and might well report on any such interaction (assuming that the probe does not sterically

hinder the interaction). This would be the method of choice in the absence of any improved BIAcore technology.

3.4 *IN VITRO* MOTILITY ASSAYS.

All *in vitro* motility assays were carried out in collaboration with Dr Justin Molloy (University of York, UK). The assay system used measures actin filament speed; no allowance was made for the direction of movement. The system is well established with myosin and HMM; less work has been done with S1 and a comparison of S1(A1) and S1(A2) has not been reported. Under conditions which are used routinely in Dr Molloy's laboratory for the study of myosin and HMM (described in section 2D.2) both isoenzymes moved actin at a detectable speed - although this speed was an order of magnitude less than that for HMM. (Histograms showing the range of filament speeds is shown in figure 3.8; the data are summarised in the table at the end of this section). However, the filament speeds for the two isoenzymes were not significantly different (Student's t-test gives t=1.9; P lies between 5 and 10 %).

The ionic strength at which this assay was carried out is approximately equivalent to 40 mM sodium chloride - much higher than that used for the MgATPase assays with these isoenzymes. Since kinetic differences between the isoenzymes are only apparent at low ionic strength (Pope *et al.* 1981), it is perhaps not surprising that no differences in motility could be detected at this ionic strength. Reduction of the ionic strength (to approximately 20 mM, in buffer containing no added potassium chloride and 13 mM imidazole hydrochloride, pH 7.4, 2 mM magnesium chloride, 0.5 mM EGTA, 1.5 mM ATP), resulted in much reduced motility, reflecting, perhaps the tighter binding between S1 and actin at low ionic strength (Katoh and Morita, 1996). This reduced movement made it difficult to establish whether the motion represented genuine motility or noise resulting from the instrumentation, unequal photobleaching of different parts of the actin filament and Brownian motion of the filaments. The lower speed with S1(A1) although statistically significant (t=8.9; P<0.1%) may well represent reduced Brownian motion resulting from this isoenzyme's stronger affinity for F-actin (Winstanley *et al.* 1977). Examination of the paths of filaments under these conditions show that the

motion is essentially random compared to the more directional motion seen at higher ionic strength (figure 3.9).

It is interesting that lowering the ionic strength reduces the filament speed. This is in contrast to reports of the opposite effect on the MgATPase activity of S1 (Bottomley and Trayer, 1975). The sliding velocity is however a function of k_{cat} (Spudich, 1994) rather than the activity (which, of course, varies with actin concentration). k_{cat} increases with ionic strength as does the apparent K_M for actin (Wagner *et al.* 1979). It is this latter fact which explains the apparent discrepancy: at lower actin concentrations (as normally used in MgATPase assays), the shift of the activity *vs* [actin] curve to the right at higher ionic strengths will result in lower activities. However, at saturating actin concentrations, the activity will be greater at higher ionic strengths (figure 3.10).

Before any work can be done with this system to investigate the effects of various ELC mutations on filament sliding speed, conditions under which S1(A1) and S1(A2) can be reproducibly distinguished must be found. This work suggests ways in which this might be achieved. It is possible that, at low ionic strength, the filaments are moving, but that photobleaching is occurring before this movement is detected. The solution to this problem is to use a timed shutter system where the filaments are illuminated at regular intervals, but only for long enough to record an image. This would allow the filaments to be tracked for much longer periods of time (photobleaching occurred after about 20 seconds of continuous illumination). Alternatively, ionic strengths intermediate to those used here could be tried in order to find conditions under which the filaments move at a detectable rate, but at significantly different speeds, depending on the isoenzyme.

Motor	Conditions	Mean filament speed / $\mu m.s^{-1}$	Standard deviation	Number of filaments analysed	Total number of data points
S1(A1)	Standard	0.29	0.13	25	297
S1(A2)	Standard	0.31	0.11	16	227
S1(A1)	Low ionic strength	0.10	0.062	12	127

S1(A2)	Low ionic strength	0.18	0.091	13	165
HMM	Standard	1.88	0.76	10	62

3.5 DISCUSSION.

The results presented in this chapter show that an actin binding site in the first eleven residues of A1-type ELCs modulates the actin activated MgATPase kinetics of S1. The actin binding site in the HmAtELC could be located even more precisely by making successive deletions from the N-terminus. The correlation between actin binding and kinetic modulation was established for the first time using a protein engineering approach. Some of the advantages of a recombinant source of A1-type ELC are discussed in section 7.1. Several methods for probing the rôle of this light chain were developed in the course of this work. A protocol for cross-linking the ELC (both free and bound to the heavy chain of S1) to F-actin was developed and successfully applied. This method proved to be of great value in the assessment of other HmAtELC mutants (Chapters 4 and 5) and in studies on the RLC from *Drosophila* intermediate flight muscle (Chapter 6). The use of a surface plasmon resonance based biosensor to monitor the ELC-actin interaction proved unsuccessful due to short-comings in the technology. Although both S1 isoenzymes moved actin in an *in vitro* motility assay, conditions under which the two could be distinguished were not found. However, solutions to the problems encountered both with the BIAcore and the *in vitro* motility assays were suggested by the results obtained and are discussed in the appropriate sections.

Chapter Four:

Rôle of the Proline Rich Region in A1-type Myosin Essential Light Chains.

4.1 Introduction.

The N-terminal eleven (or fewer) residues of A1-type ELCs forms an actin binding site which is responsible for modulating the actin activated MgATPase activity of myosin S1 (Chapter 3). This poses questions about the function of the sequence immediately C-terminal to this. Previous work suggests that this proline-rich region adopts a rigid, rod-like structure (Bhandari *et al.* 1986; Abillon *et al.* 1990) which may be responsible for bridging the gap between the ELC and actin. This hypothesis was tested by altering the length of this region and assessing the mutants' ability to modulate the actin activated MgATPase kinetics of S1 and to bind to actin both in free solution and complexed to the heavy chain. The results from this work not only answered the original question, but also provided the basis for informed speculation about how information is transmitted in the acto-S1 complex.

4.2 Paper II: "Rôle of the Proline Rich Region in A1-Type Myosin Essential Light Chains: Implications for Information Transmission in the Acto-Myosin Complex."

This paper was submitted to FEBS Letters and is currently undergoing review.

The Rôle of the Proline-Rich Region in A1-type Myosin Essential Light Chains: Implications for Information Transmission in the Actomyosin Complex.

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ABSTRACT

The proline-rich region of A1-type myosin essential light chains functions as a spacer arm separating an actin binding site at the extreme N-terminus from the remainder of the protein. Alteration of the length of this region leaving the actin binding site intact results in altered actin-activated MgATPase kinetics when these light chains are hybridised into myosin subfragment-1. In the case of a mutant in which the length of the proline-rich region was doubled, actin binding by the light chain was uncoupled from kinetic modulation. The implications of this result for information transmission in the actomyosin complex are discussed.

Keywords: Muscle contraction, actin binding, proline, protein engineering, ATPase, protein-protein interaction.

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Abbreviations: S1, myosin subfragment-1; S1A1, S1A2, S1 containing alkali 1 (A1) or alkali 2 (A2) essential light chain; RLC, myosin regulatory light chain; ELC, myosin essential light chain; HmAtELC, human atrial ELC; HmAtELCΔXP, HmAtELC lacking the proline-rich region; HmAtELC2XP, HmAtELC with a proline rich region twice the

length of the wild type; EDC, 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide; F-actin, filamentous actin; Xxx, any amino acid; TEA.HCl, triethanolamine hydrochloride; $A_{280,1mg/ml}$, absorbance at 280 nm of a 1 $mg.ml^{-1}$ solution.

1. Introduction.

It has now been shown unequivocally that A1-type myosin essential light chains (ELC) bind actin and that this actin binding event is directly correlated to modulation of the actin activated MgATPase kinetics of the myosin motor [1]. A1-type ELCs of vertebrate striated muscle are distinguished by the presence of 40 or so amino acids at the N-terminus which are not present in the alternative isoform - A2-type [2]. In vertebrate fast skeletal muscle, the two proteins result from differential splicing of the same mRNA transcript [3].

The crystal structure of myosin subfragment 1 (S1, a soluble, proteolytic fragment of myosin which retains the essential motor properties of the intact molecule [4]) shows that the light chains (there are two per S1 molecule: one ELC and one regulatory light chain, RLC) are far (>8 nm) from the site of MgATP hydrolysis and the main sites of actin binding which are located on the heavy chain of S1 [5-7]. Nevertheless, events in this light chain binding domain can have profound effects on the behaviour of the myosin motor. In scallop myosin, calcium ion binding by the ELC switches the motor on [8] and in smooth muscle myosin, phosphorylation of the RLC results in the switching on of the motor (reviewed in [9]). In both cases, a message must pass from the light chain to the distant motor domain.

Although the light chains are not responsible for regulating myosin from striated muscle, they can modulate the kinetics of the motor. A1-type ELCs typically endow S1 carrying this light chain (S1A1) with a lower apparent K_M for actin and a lower k_{cat} for MgATP turnover in the presence of F-actin than A2-type ELCs [10,11]. This suggests that A1-type ELCs increase the affinity of S1 for actin (a result supported by F-actin affinity chromatography studies[12,13]) but slow down the molecular motor. We have

recently demonstrated that this increase in affinity results from a direct ELC-actin interaction involving the first eleven residues of the human atrial ELC (HmAtELC, an A1-type ELC [14]) and that removal of these residues results not only in loss of actin binding by the light chain but also in loss of kinetic modulation in S1 molecules carrying it [1]. However, these studies did not address the problem of how the ELC - which is some 8 nm from the actin filament in models of the acto-S1 complex [6,7] - can contact actin. In order to explain the apparent contradiction between our work and the structural work, it is necessary to explain how this gap can be bridged.

The remaining 30-odd residues in the extension on A1-type ELCs is rich in (Xxx-Pro) repeats (where Xxx represents any amino acid; lysine and alanine are most commonly found, Figure 1). Such sequences form rigid extended structures [15-18] and we tested the hypothesis that this sequence is responsible for bridging the gap between the ELC and actin which is apparent from the models of acto-S1 derived from the crystal structures. To do this, we constructed mutants of the HmAtELC (which we have previously expressed in *E. coli*) in which the length of this region was altered. The effect of these changes was assessed by hybridising the mutant light chains into rabbit skeletal S1 and testing whether the MgATPase kinetics were S1A1- or S1A2-like. The ability of the mutants to bind actin - both in complex with the heavy chain and "free" in solution - was tested by chemical cross-linking. We found that a mutant with this region deleted (HmAtELCΔXP) and one in which the length was doubled (HmAtELC2XP) endowed kinetics on their respective S1 hybrids which were significantly different from the wild type HmAtELC. The actin binding ability of both mutants is not compromised by altering the length of the putative spacer arm: both can be cross-linked to actin when in free solution. However, only HmAtELC2XP and the wild type HmAtELC can cross-link when hybridised into S1.

These results argue strongly for the rôle of the (Xxx-Pro) rich region being to position the actin binding site correctly on the surface of actin. Furthermore, the results from the HmAtELC2XP mutant hybrid give rise to speculation on the pathways of information transfer within the acto-S1 complex.

2 Experimental

2.1 Construction of plasmids carrying genes encoding mutant HmAtELCs.

HmAtELCΔXP (consisting of residues 1 to 11 of the wild type sequence [14] fused directly to residue 45 (Figure 1)) was expressed from a DNA sequence constructed by PCR [19] amplification of the wild type HmAtELC cDNA (a gift from Dr Anna Starzinski-Powitz, University of Cologne, Germany) using a 3′-primer (5'-AAGGATCCAGACTCTGCTTCACCCTG-3') which complements the 3′ end of the gene and introduces a *BamHI* site 3′ to the stop codon along with a 5′-primer (5'-AAACATATGGCTCCCAAGAAGCCTGAGCCTAAGAAGGAGGCAGACTTC ACTGCCG-3') which complements bases corresponding to amino acid residues 44 to 50 and introduces a sequence coding for the first 11 amino acids, an initiation (methionine) codon and an *NdeI* restriction site 5′ to this. The identity of this PCR product was checked by exploiting the fact that an *NdeI-ApaI* restriction fragment which spans the deletion is reduced from 150 bp in the wild type to 49 bp in the mutant. The PCR product was then subjected to a double restriction enzyme digest using the enzymes *NdeI*/*BamHI* and ligated into *NdeI*/*BamHI* cut pET-11c expression vector [20] using standard molecular biology techniques [21].

HmAtELC2XP (consisting residues 1 to 32 of the wild type sequence followed by a repeat of residues 15 to 32 and the wild type sequence thereafter) was constructed by cutting pET-11c with the wild type HmAtELC sequence inserted between the *NdeI*/*BamHI* sites in the vector with the restriction enzyme *Bsu36I* which cuts uniquely at nucleotide position 92 (which forms part of the codon coding for amino acid 30). This permits the insertion of pre-annealed, synthetic oligonucleotides with sequence corresponding to amino acid residues 15 to 32 with ends which complement the overhanging ends resulting from the restriction enzyme digest (5'-TGAGCCAGCTCCAGCTCCAGCTCCAGCTCCTGCACCAGCCCCTGCCCCA GCTCC-3' and

5’-TCAGGAGCTGGGGCAGGGGCTGGTGCAGGGGCTGGAGCTGGAGCTGGAG CTGGC-3’) into the *Bsu36I* cut pET-11c-HmAtELC construct.

Both mutant sequences were verified by double stranded DNA sequencing [22].

2.2 Protein Preparations.

All protein manipulations were carried out at 4°C. Actin [23] and chymotryptic myosin S1 isoenzymes [10] were prepared according to published procedures. Note that chymotrypsin cuts myosin between the ELC and RLC binding sites on the heavy chain resulting in an S1 that has only the ELC associated with it. Recombinant, mutant ELCs were prepared by exactly the same method as the wild type HmAtELC which has been described previously [1], and their molecular masses characterised by matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS).

F-actin was prepared by reconstituting freeze dried G-actin in buffer A (5 mM triethanolamine hydrochloride (TEA.HCl), pH 7.6, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.25 mM DTT) in the presence of 1 mg solid DTT. This solution was dialysed vigorously overnight against buffer A, insoluble matter removed by centrifugation at 80000g for 30 minutes and polymerisation to F-actin initiated by the addition of $MgCl_2$ (2 mM, final concentration).

2.3 Discontinuous actin activated MgATPase assays.

Prior to analysis, the HmAtELC mutants were hybridised into rabbit skeletal S1A2 using the ammonium chloride dissociation method [11] as modified in [24] and the pure hybrid isolated from free light chains and residual S1A2 by ion exchange chromatography on SP-Trisacryl™ (IBF Biotechnics, France) [25]. Pure hybrid was collected and concentrated by precipitation in 70%(w/v) ammonium sulphate. Precipitates were taken up in 5 mM TEA.HCl, pH 7.5, 0.25 mM DTT and dialysed against this buffer overnight.

Assays were performed in 5 mM TEA.HCl at 25°C essentially as described previously [1]. Briefly, S1 hybrids were mixed with F-actin dissolved in buffer A supplemented with 2 mM $MgCl_2$. S1 concentrations varied between 0.06 μM and 0.16 μM. Reactions were initiated by the addition of $MgCl_2$ to a final concentration of 2.5 mM and ATP to a concentration of 2.0 mM and allowed to proceed for 10 minutes after which time the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 6.7%(w/v). The amount of phosphate released in this time was quantified spectrophotometrically [26]. Rates of phosphate release (in μmol P_i released s^{-1}) were normalised by dividing by the S1 concentration. These activities were plotted against actin concentration and the kinetic parameters (apparent K_M for actin and k_{cat} for MgATP turnover) were derived by non-linear curve fitting to the equation Activity = (k_{cat}.[actin])/(K_M + [actin]) using the program Fig-P for Windows (Biosoft, Cambridge, UK). All points were weighted equally.

2.4 Cross-linking.

Cross-linking was carried out using the zero-length cross-linker EDC. Proteins were mixed in the concentration ratios indicated below and incubated at room temperature prior to cross-linking. Protein concentrations ranged between 10 μM and 40 μM. EDC was then added to a final concentration of 67 mM and the reaction mixture left for a further hour before addition of 2%(w/v) SDS and 5%(v/v) β-mercaptoethanol to terminate the reaction before analysis by SDS-PAGE. Bands were identified by reference to previous work [1] and by reference to molecular mass standards.

2.5 Analytical methods.

SDS-PAGE analysis was performed using 12% slab gels run in 0.1 M tris-bicine, 0.1%(w/v) SDS at 50 mA (constant current) [27]. Bands were visualised by staining with Coomassie blue. MALDI-MS [28] was carried out by Mr P Ashton, School of Chemistry, The University of Birmingham, UK. Concentrations were estimated by UV spectroscopy using the following values: $A_{280,1mg/ml}$(S1) = 0.80 mg^{-1}.ml;

$A_{280,1mg/ml}$(ELC) = 0.22 mg^{-1}.ml; $A_{290,1mg/ml}$(actin) = 0.63 mg^{-1}.ml and molecular masses: HmAtELC, 21 kDa; HmAtELCΔXP, 18 kDa; HmAtELC2XP, 24 kDa; actin 42 kDa.

3. Results.

3.1 Kinetic assays show that neither mutant behaves like the wild type.

The results of steady state kinetic analysis of both mutant hybrids are shown in Figure 2 and the kinetic constants derived from this analysis are shown in Table 1. The kinetic parameters for rabbit skeletal S1A1 and S1A2 and the S1(HmAtELC) hybrid under the same conditions are also given. The S1(HmAtELCΔXP) hybrid clearly has A2-like kinetics, showing that the actin binding site, although intact, is unable to influence the myosin motor. The results with the S1(HmAtELC2XP) hybrid are more interesting. The hybrid shows a high (ie S1A2-like) k_{cat} but a low (ie S1A1-like) apparent K_M for actin, suggesting that this hybrid interacts with actin with similar affinity to the wild type hybrid, yet it turns MgATP over at an S1A2-like rate.

3.2 The actin binding site of both mutant light chains is functional.

It was important to ascertain that changing the length of the (Xxx-Pro) rich region, while leaving the actin binding site intact does not affect the functionality of this site. The wild type protein can be cross-linked to F-actin [1] and we tested the ability of both mutants to bind in the same way. Both clearly do (Figure 3), showing that the actin binding site is fully functional and unaffected by changes occurring C-terminal to it.

3.3 The length of the (Xxx-Pro) rich arm controls actin binding by the mutants in the hybrids.

Rabbit skeletal A1-type ELC can be cross-linked to actin when in complex with the heavy chain [29-31], as can HmAtELC in S1(HmAtELC) (Figure 4a). However, if the (Xxx-Pro) rich region is responsible for positioning the actin binding site then testing for this cross-linking will be a sensitive probe for whether this site is correctly located in the acto-hybrid S1 complex. HmAtELCΔXP in S1(HmAtELCΔXP) cannot be cross-linked to F-actin (Figure 4b), despite our success in cross-linking the free mutant light chain (section 3.2). Thus, we conclude that this actin binding site is too far from actin to interact.

On the other hand, HmAtELC2XP in complex with rabbit skeletal heavy chain *can* be cross-linked to F-actin (Figure 4c). This result is in agreement with the kinetic data on this hybrid which suggests that it binds to F-actin as strongly as the wild type does. This mutant hybrid is particularly interesting because although actin binding (as indicated by positive cross-linking results and low apparent K_M for actin) is correlated to modulation of k_{cat} in the wild type [1] these two activities of the ELC have become uncoupled in this case.

4. Discussion.

4.1 General

The (Xxx-Pro) rich region of A1-type ELCs does function as a spacer arm in S1 hybrids: reduction of its length prevents the actin binding site at the N-terminus of the light chain making contact with actin and this results in S1A2-like actin activated MgATPase kinetics. Increasing the length of the arm also affects the kinetics of the resulting hybrid.

4.2 Why a rigid spacer arm?

It is clear from models of acto-S1 based on the crystal structures [6,7] that a large gap needs to be bridged between the ELC and the actin filament if the two are to make

contact. None of the residues in the N-terminal extension of the A1-type ELC are observed in this structure [5] and it is entirely feasible that the "missing" residues could bridge the gap. However, to do so they would have to be in an extended chain.

For most sequences this would not be the natural state and actin binding would be accompanied by a transition from a folded to an extended structure by the "bridging" sequence. This transition would be energetically unfavourable for not only would it involve the breaking of many intra-molecular contacts (enthalpically unfavourable) it would also require the imposition of greater order onto the system (entropically unfavourable). Both these unfavourable influences on the free energy of binding can be overcome by having a sequence (such as the one found in A1-type light chains) which is elongated and rigid *prior* to binding [15-18]: the enthalpic costs need never be paid and the entropic costs will already have been (largely) paid [32].

There is an alternative (but equivalent) kinetic way of approaching this problem. If the actin binding site were on a flexible arm, its search for its target site on actin would (to a first approximation) be one through three-dimensional space. By fixing the actin binding site on the end of a rigid arm this is reduced, essentially, to a two dimensional search over a limited area. (In other words we would expect the principal benefits of having a rigid arm would be an increased association rate constant; the dissociation rate constant should be relatively unaffected.)

4.3 Pathways of communication in the actomyosin complex.

The site of interaction between the ELC and actin is some 8 nm from the site of MgATP hydrolysis [6,33] and furthermore, the ELC and the myosin heavy chain interact with different actin monomers - most likely the ELC interacts with the monomer immediately adjacent to the one the heavy chain contacts [1,31]. There are, therefore, two pathways by which information could be transmitted from the ELC-actin interaction site to the active site: one proceeding *via* S1 and the other passing through the actin filament.

The results with S1(HmAtELC2XP) hybrid favour one pathway over the other. In this hybrid, the ELC interacts with actin in a similar manner to the wild type (hence the S1A1-like apparent K_M for actin and the success in cross-linking the light chain to actin in the hybrid) and yet, the message fails to reach the active site. This argues against the "through S1" pathway - since it is unlikely that this pathway could distinguish between different modes of actin binding: it would be an on/off switch triggered by the binding event. On the other hand, if the additional length of the arm means that the ELC contacts a monomer yet more distant from the sites of heavy chain contact with actin than the wild type (and this seems likely on account of the considerably increased length of the mutant arm) then it is conceivable that this message might "fade out" as it travels that extra distance within the actin filament.

This would suggest that information transmission in this system proceeds by a different pathway than in thick filament regulated smooth muscle myosin. Since there is no opportunity for light chain-actin interaction in this system, information must pass through the myosin molecule. Although smooth muscle RLC is phosphorylated near the N-terminus, the C-terminus appears to be most important in initiating information transmission to the active site [34]. The pathway does not require the ELC [35] suggesting that the initial stages of information transfer involve the N-terminus of the RLC, then the C-terminus and thereafter the myosin heavy chain.

Clearly the acto-myosin system is a complex one - acted upon by many, interacting modulators. It seems likely that different pathways of communication are important in different systems. While determining the rôle of the proline-rich region of A1-type myosin essential light chains we have also been able to suggest what one important pathway in striated muscle myosin might be. A1-type myosin ELCs bind actin and modulate motor function; they do so by transmitting a message to the active site of myosin. Our results suggest that this message is unlikely to pass through S1 and may well be propagated through the actin filament. The possession of a mutant ELC which, when hybridised into S1, results in the uncoupling of the actin binding and kinetic modulation functions of the light chain should enable this important problem to be investigated further.

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Hybrid etc	Apparent K_M for actin / μM	k_{cat} / s^{-1}	n
RbSk S1A1	4.2±0.6	13.7±1.1	20
RbSk S1A2	31.9±9.9	34.6±9.5	24
S1(HmAtELC)	5.4±1.5	15.3±2.8	32
S1(HmAtELCΔXP)	26.6±4.8	24.3±3.8	24
S1(HmAtELC2XP)	5.7±0.7	28.3±2.7	28

Table 1: Kinetic parameters of the mutant hybrids. Rabbit skeletal (RbSk) S1A1 and S1A2 and the wild type HmAtELC hybrid under the same conditions (which have been reported previously [1]) are given for comparison. n is the number of determinations.

FIGURE LEGENDS

Figure 1: Sequences of the wild type and mutant HmAtELCs. Gaps have been introduced to maximise sequence identity. Numbering is based on the wild type HmAtELC sequence [14], neglecting the N-terminal methionine residue. Only the first 99 residues are shown; the sequences are identical thereafter.

Figure 2: Actin activated MgATPase kinetics of the wild type and mutant hybrids. Squares, S1(HmAtELC); circles, S1(HmAtELCΔXP); triangles, S1(HmAtELC2XP). The lines were fitted to each data set as described in the text.

Figure 3: Cross-linking of mutant HmAtELCs in free solution to F-actin. M, molecular mass markers; lanes 1-3, HmAtELCΔXP:actin 1:2, 1:1, 2:1 respectively; lane 4, HmAtELCΔXP; lanes 5-7, HmAtELC2XP:actin 1:2, 1:1, 2:1 respectively; lane 8

HmAtELC2XP; lane 9; actin. EDC was added in all cases. A, HmAtELCΔXP; B, HmAtELCΔXP-actin; C, HmAtELC2XP; D, HmAtELC2XP-actin.

Figure 4: Cross-linking of wild type and mutant HmAtELC hybrids to F-actin.
Figure 4a: Lanes 1 and 2, S1(HmAtELC):actin 1:4 and 1:2 respectively; lane 3, S1(HmAtELC).
Figure 4b: Lanes 1 to 3, S1(HmAtELCΔXP):actin 1:4, 1:2 and 1:1 respectively; lane 4 actin; lane 5, HmAtELCΔXP.
Figure 4c: Lanes 1 and 2, S1(HmAtELC2XP):actin 1:4 and 1:2 respectively; lane 3, S1(HmAtELC2XP).
EDC was added in all cases.

4.3 DISCUSSION.

The experiments described in Paper II show clearly that the rôle of the proline rich region of the HmAtELC is to position the actin binding site correctly on the surface of actin. The question of how information is transmitted within the acto-S1 complex will be discussed further in section 7.2.2. Although these experiments prove that the length of the arm is important they do not show how tolerant the system is to smaller changes in length, nor do they assess the requirement for the arm to be rigid. The length of the arm could be progressively reduced and the effects on the MgATPase activity of the resulting hybrids assessed. The proline-rich region could be substituted (all or in part) for a more flexible sequence (eg $(AlaGly)_n$) and again the effects on the actin activated MgATPase kinetics assessed. In both sets of experiments the ability of the light chain to bind actin (both free and in hybrid) should be assessed by cross-linking (as described in Paper II). The value of these experiments would be greatly enhanced if the binding of the mutant hybrids to actin could be quantified (see section 3.3.3).

Chapter Five:

Size and Charge Requirements for Actin Binding and Kinetic Modulation by A1-type myosin ELCs.

5.1 INTRODUCTION.

Access to recombinant A1-type ELC and knowledge about the location of the actin binding site within it gives us the opportunity to ask - and answer meaningful questions - about the rôle of individual amino acids in both actin binding and kinetic modulation. NMR evidence (from the full length rabbit skeletal A1-type ELC) (Henry *et al.* 1985a; Henry *et al.* 1985b; Trayer *et al.* 1987) suggests the involvement of one of more lysine residues and the N-terminal trimethylalanine group. Unpublished data (HR Trayer and KJ Smith) highlight the importance of the trimethylalanine residue and the lysine residue at position 4 (which is conserved throughout A1-type ELCs, figure 5.1). Initial mutagenesis experiments concentrated on these residues. The size of the side chain at position 1 was altered and a range of alterations (Arg, Ala and Asp) were made at position 4 to probe the requirement for the charge at this site. These studies were successful and were extended to investigate the rôle of lysine 3 (which is also well conserved, figure 5.1).

5.2A PAPER III: "SIZE AND CHARGE REQUIREMENTS FOR KINETIC MODULATION BY A1-TYPE MYOSIN ESSENTIAL LIGHT CHAINS"

The following paper was submitted to the European Journal of Biochemistry and was undergoing review at the date of thesis submission.

Size and Charge Requirements for Kinetic Modulation and Actin Binding by A1-type Myosin Essential Light Chains.

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Abbreviations: S1, myosin subfragment 1; S1(A1), S1(A2) myosin subfragment one carrying A1-type or A2-type essential light chains respectively; ELC, myosin essential light chain; HmAtELC, human atrial myosin essential light chain; RLC, myosin regulatory light chain; TEA.HCl, triethanolamine hydrochloride; DTT, dithiothreitol.

SUMMARY

There are two forms of essential light chain (ELC) in vertebrate striated muscle: A1-type and A2-type. Consequently there are two isoenzymes of myosin subfragment 1: S1(A1) and S1(A2) depending on which ELC is associated with the heavy chain. A1-type ELCs have an additional 40 or so amino acid residues at the N-terminus compared to the A2-type. These additional amino acids in A1-type ELCs are responsible for binding actin and modulating the kinetic activity of myosin molecules that carry them. The requirements for actin binding and kinetic modulation of the myosin motor by an A1-type ELC were been investigated by a protein engineering approach. Mutations in the actin binding site of the human atrial ELC (HmAtELC) result in altered actin activated MgATPase kinetics when the recombinant light chains are hybridised into rabbit skeletal myosin subfragment 1. Substitution of the positively charged lysine residue at position 4 with the neutral amino acid alanine results in decreased kinetic modulation (the resulting hybrid S1 has kinetic parameters approximately midway

between S1(A1) and S1(A2) under the same conditions) and decreased actin binding (as judged by chemical cross-linking). Replacement of this residue with aspartate (negatively charged) reduces actin binding still further and the hybrid S1 is almost S1(A2)-like in its kinetic parameters. Substitution of lysine 3 for alanine gives similar results to same replacement at position 4. However, the size of the side chain at position 1 is unimportant: alteration of the wild type amino acid (alanine) to valine results in no change in the kinetic parameters of the hybrid S1 or in the ELC's ability to bind actin. Furthermore we found a statistically significant positive correlation between the apparent K_M for actin and the k_{cat} for MgATP turnover for each mutant hybrid. This strengthens our belief that the binding of actin by A1-type ELCs results directly in modulation of the myosin motor.

Keywords: Molecular motor protein, actin binding, protein engineering, protein-protein interaction, muscle contraction.

INTRODUCTION.

Muscle contracts when a molecular motor protein, myosin, hydrolyses MgATP and uses the energy so released to pull past filaments of its partner protein, actin. Although the mechanism of contraction appears to be broadly similar between muscle types, the way in which it is controlled is not. The universal trigger for contraction is a rise in the intracellular free calcium concentration, but the way in which this is sensed and the way in which the message is transmitted to the active site of myosin varies considerably. Furthermore, many muscle systems are modulated by additional proteins which fine tune the on/off signaling resulting from calcium ion concentration fluctuations.

The regulation of scallop myosin is the simplest known system. Calcium ions bind directly to the essential light chain (myosin is a hexamer of two 200 kDa heavy chains, two essential light chains, ELC, of 17 to 21 kDa and two regulatory light chains, RLC, 20 kDa) and this acts as a direct switch for the actin activated MgATPase of the motor [1,2]. In smooth muscle myosin the situation is more complex: calcium ions bind to calmodulin which in turn activates myosin light chain kinase. The kinase transfers a

phosphate group from MgATP to the RLC - an event which triggers activation of the motor (reviewed in [3,4]). The light chains of skeletal and cardiac muscle are not involved in the primary regulation of muscle contraction. This is achieved through calcium ion binding by the troponin/tropomyosin complex located on the actin filament (reviewed in [5]).

However, the ELC of vertebrate striated muscle exists in two isoforms: A1-like and A2-like [6,7] and the presence of one, or the other isoform appears to fine tune the contractile properties of the myosin molecule carrying it. This effect has been demonstrated in single muscle fibres [8], in *in vitro* motility assays [9] and in enzyme assays [10], where experiments are generally carried out with the more biochemically amenable myosin subfragment 1 (S1, a soluble, proteolytic fragment of myosin which retains the actin binding and MgATPase activities of the intact molecule). S1 prepared by chymotryptic digestion carries only one ELC and a fragment of the heavy chain [10]. Thus there are two possible S1 isoenzymes - S1(A1) and S1(A2) - depending on which ELC isoform is associated with the heavy chain.

Recent mutagenesis studies have established that the extreme N-terminal residues of A1-type ELCs (which differ from A2-like ELCs by having 40-odd additional amino acid residues at the N-terminus) encode an actin binding site. The site lies within the first 11 residues and actin binding by these residues results directly in modulation of the kinetic properties of S1 (DJT and IPT, manuscript submitted). The remaining residues of the N-terminal extension of A1-type ELCs are highly proline rich and form an extended structure [11] responsible for correctly positioning the actin binding site on the surface of actin. Access to a recombinant source of A1-type ELC (the human atrial form, HmAtELC [12]) enables us to ask - and answer - specific questions about the role of individual amino acids in the modulatory event.

NMR studies on the intact rabbit skeletal A1-type ELC suggest the involvement of one or more lysine residues and the N-terminal trimethylalanine residue in actin binding [13-15]. Given the close correlation between actin binding and kinetic modulation we concentrated initially on the N-terminal residue and on the lysine residues at positions 3

and 4. The importance of size at position 1 was investigated by mutating alanine 1 to valine. This resulted in no significant change in the actin activated MgATPase kinetics of the resulting hybrid S1, suggesting that the size of this side chain is unimportant. On the other hand, altering the charge at either position 3 or position 4 produced significant changes in both the apparent K_M for actin and the k_{cat} for MgATP turnover for S1 hybrids carrying these mutant ELCs. Indeed reduction of the positive charge at either position gave a kinetic phenotype which was more S1(A2)-like than the wild type hybrid (which is S1(A1)-like (DJT and IPT, manuscript submitted)). These studies show that the k_{cat} is directly controlled by the apparent K_M for actin further supporting the view that actin binding by A1-type ELCs results directly in kinetic modulation of the myosin motor.

EXPERIMENTAL PROCEDURES.

Production of expression vectors carrying mutant HmAtELCs.

All mutant proteins were expressed from plasmids derived from the T7-based over-expression vector pET-11c [16]. The polymerase chain reaction [17] was used to amplify the HmAtELC gene (kindly supplied to us by Dr A Starzinski-Powitz, University of Cologne, Germany in the vector pUC-19) and the various mutations were introduced by using different 5' oligonucleotide primers which incorporated changes in the appropriate codons. In all cases the primers were designed so as to introduce an *NdeI* restriction enzyme site 5' to the coding sequence and a *BamHI* site 3' to it. PCR products were digested with these two enzymes and the product of this reaction ligated into *NdeI*/*BamHI* cut pET-11c. Plasmids containing these inserts were transformed into *E. coli* BL21(DE3) cells for protein over-expression as previously described for the wild type HmAtELC (DJT and IPT, manuscript submitted). Over-expression was monitored by comparing the SDS-PAGE profiles of cell extracts from cells before and after induction with IPTG. Expression was judged successful if an additional band of approximately the correct molecular mass appeared after induction. The sequences of all mutant ELC constructs were verified by double stranded dideoxynucleotide sequencing [18] and the N-terminal sequence checked by amino acid sequencing.

Figure 1 shows the amino acid sequence of the first 10 residues of the wild type and mutant proteins.

Protein preparations.

Unless otherwise stated all protein manipulations were carried out at 4°C. Actin was prepared from muscle acetone powder according to standard techniques [19]. Actin was stored as G-actin freeze dried from buffer A (5 mM triethanolamine hydrochloride (TEA.HCl), pH 7.5, 0.2 mM calcium chloride, 0.2 mM ATP, 0.25 mM dithiotheitol (DTT)) at -20°C until required. F-actin was prepared by reconstituting this freeze dried powder in buffer A and dialysing vigorously overnight. The resulting G-actin solution was clarified by centrifugation at 80000g for 30 minutes and polymerisation to F-actin induced by the addition of magnesium chloride to a final concentration of 2.0 mM. Chymotryptic myosin S1 (which contains one mole intact ELC per mole S1, but no RLC) was prepared according to the method of Weeds and Taylor [10], except that SP-Tris Acryl™ (IBF Biotechnics, France) was used to separate the isoenzymes (S1(A1) and S1(A2)) [20]. Aliquots of the purified isoenzymes were shell frozen at -80°C in the presence of 4 mg sucrose per mg S1 and 2 mM DTT and stored at -70°C until required.

Recombinant ELCs were purified by ammonium sulphate fractionation and column chromatography on DEAE-Sepharose Fast-Flow (Pharmacia) exactly as described previously for the wild type HmAtELC (DJT and IPT, manuscript submitted). Purified ELCs were stored as freeze dried powders at -20°C until required.

Steady state MgATPase assays.

The effect of each mutation on the steady state MgATPase kinetics of S1 was assessed by hybridising the light chain into rabbit skeletal S1(A2) using the ammonium chloride dissociation method [7] as modified in [21] and using SP-Trisacryl to separate the mutant hybrid from the residual S1(A2) [20]. Hybrids were concentrated by

precipitation in 70%(w/v) ammonium sulphate before being taken up in about 1 ml 5 mM TEA.HCl, pH 7.5, 0.25 mM DTT and dialysed against this buffer overnight.

MgATPase activities were determined by measuring phosphate release in a stopped enzyme assay at 25°C. Various concentrations of F-actin (in buffer A supplemented with 2.0 mM magnesium chloride; the amount of supplemented buffer A plus F-actin solution was kept constant at 40 µl per ml assay mix) were mixed with mutant hybrid S1s (concentrations of which varied between 0.04 µM and 0.17 µM) in 5 mM TEA.HCl, pH 7.5, 0.25 mM DTT. The reaction was initiated by addition of magnesium chloride to 2.5 mM and ATP to 2.0 mM.

At the end of the reaction (typically 6 to 10 minutes after initiation), the reaction was stopped with 6.7%(w/v) (final concentration) trichloroacetic acid, the precipitated proteins removed by centrifugation and the amount of inorganic phosphate determined spectrophotometrically [22]. In all cases, a control reaction containing no actin was set up to allow for the low non-actin activated MgATPase activity of S1. The rate (in µmol P_i released s^{-1}) was converted to MgATPase activity by dividing by the concentration of hybrid S1 in the assay. Activities were plotted against actin concentration and the apparent K_M for actin and the k_{cat} for MgATP turnover were determined by non-linear curve fitting to the equation activity = $(k_{cat}.[actin])/(K_M + [actin])$ using the program FigP for windows (Biosoft, Cambridge, UK). All points were weighted equally.

Cross-linking.

Recombinant light chains were reconstituted by dissolving in 5 mM TEA.HCl, pH 7.5, 0.25 mM DTT in the presence of 1 mg solid DTT per mg freeze dried powder and 10 µl 1 M sodium hydroxide per 1 ml ELC solution. The resulting solutions were then dialysed overnight against 5 mM TEA.HCl, pH 7.5, 0.25 mM DTT and any insoluble matter then removed by centrifugation at 15000g for 15 minutes. F-actin was prepared

as described above. Mutant ELCs and actin were mixed at the molar ratio 1:1 (concentration of each protein, 20 μM) and left at room temperature for one hour in order to allow the two proteins to come to equilibrium. After this time, EDC was added to a final concentration of 67 mM, the reaction was mixed gently then left for another hour before being stopped by addition of SDS to 2%(w/v) and β-mercaptoethanol to 5%(v/v) and boiling for 3 minutes. Products were analysed by SDS-PAGE and identified on the basis of their size and by comparison to previously assigned bands (DJT and IPT, manuscript submitted). S1 hybrids were cross-linked to actin using a similar protocol except that S1:actin molar ratios were typically 1:4, since increasing the saturation of F-actin with S1 decreases the amount of ELC-actin product [23].

Analytical methods.

12% SDS-PAGE gels were run at between 35 and 50 mA (constant current) and stained with Coomassie blue [24]. N-terminal protein sequencing was carried out on an Applied Biosystems 473A automated protein sequencer by AltaBioscience, School of Biochemistry, the University of Birmingham. Protein concentrations were estimated from absorbance measurements using the following values: $A_{280,1mg/ml}$(HmAtELC) = 0.22 mg^{-1}.ml; $A_{290,1mg/ml}$(Actin) = 0.63 mg^{-1}.ml and molecular masses: HmAtELC, 21 kDa; actin, 42 kDa. The mutations described in this study were not expected to alter the absorption characteristics or molecular mass of the light chain significantly and so these values were used for all mutant ELCs. F-actin concentrations were expressed as the equivalent concentration of actin monomers.

RESULTS AND DISCUSSION

The size of the side chain at position 1 is unimportant.

When hybridised into rabbit skeletal S1, the HmAtELC(A1V) mutant gives actin activated MgATPase kinetic parameters which are identical to the wild type (table 1, figure 2a). Furthermore, this mutant light chain (both free and hybridised into S1) cross-links to F-actin (figure 2b). The amount of HmAtELC(A1V)-actin product

produced is similar to the amount of wild type HmAtELC product under the same conditions. Thus we conclude that the size of the side chain is unimportant in kinetic modulation and actin binding. The most plausible explanation for this is that the side chain itself is not involved in the contact with actin and thus increasing its size does not interfere with the interaction. However, NMR studies show the involvement of this residue [13,14]. Presumably some other functionality of the residue (most likely the positive charge on the N-terminus) is involved.

A positive charge at positions 3 and 4 is essential for full kinetic modulation.

The positive charge at position 4 was varied from positive (wild type HmAtELC and HmAtELC(K4R)) through neutral (HmAtELC(K4A)) to negative (HmAtELC(K4D)), and the kinetic parameters of the hybrids became progressively more S1(A2)-like (table 1, figure 3a). Interestingly, the results for HmAtELC(K4R) hybrid show a lower apparent K_M for actin than the wild type, suggesting that this ELC may have a higher affinity for actin. Abolition of the positive charge at position 3 (HmAtELC(K3A)) gives very similar results to the same change at position 4 (table 1, figure 3b).

All three mutant ELCs cross-link to F-actin (figure 3c). However, there are clear differences in the amount of cross-linked product produced: HmAtELC(K4R) cross-links to a greater extent than HmAtELC(K4A) which in turn cross-links better than HmAtELC(K4D). HmAtELC(K4A) and HmAtELC(K3A) cross-link to similar extents. The results with S1 hybrids of these mutants are similar (not shown). Such results should be interpreted with care. Cross-linking (unlike techniques such as fluorescence) does not report on the equilibrium situation. Rather it captures equilibrium complexes once formed and prevents them from dissociating. Thus the amount of cross-linked product will depend not only on the equilibrium constant between the two species, but also the efficiency of the subsequent cross-linking reaction. This will be influenced by the concentration of the cross-linker (EDC in our case and not a constant quantity as this reagent is attacked by water and other nucleophiles in the reaction mix) and the availability of suitable groups for cross-linking. However, when similar species are compared under identical conditions (the reactions shown in figure 3c were performed

in parallel with the same EDC stock solution) then qualitative predictions about the strength of interaction can be made. These results are thus in agreement with the apparent K_M values derived from kinetic analysis, which suggest that the mutant ELCs bind actin in the following order of affinity: HmAtELC(K4R) > HmAtELC(K4A) ≈ HmAtELC(K3A) > HmAtELC(K4D).

It is clear from these results, that a positive charge at both positions 3 and 4 is required for full actin binding and kinetic modulation. Indeed it is possible that actin binding requires merely a concentration of positive charge in the N-terminal region including contributions from the N-terminus and lysines 3 and 4.

The apparent K_M for actin controls k_{cat}.

If the apparent K_M for actin is plotted against k_{cat} for all these mutants then a clear correlation is seen (figure 4). The correlation is significant at the 1% level ($r^2 = 0.98$, degrees of freedom = 6). This suggests that the K_M controls k_{cat} up to a limit defined by S1(A2). This strengthens our belief that actin binding (reflected by K_M) by the wild type A1-type ELC results directly in kinetic modulation (reflected by k_{cat}): if the first is weakened, so is the second

Significance of the N-terminal trimethylalanine residue in native A1-type ELCs.

An interesting feature of this (and previous) work is that while all native A1-type ELCs that have been analyzed [13] contain the unusual N-terminal blocking group trimethylalanine and our recombinant light chains are not post-translationally modified in any way, this apparently mades very little difference to the kinetics of S1 and S1 hybrids as we analyse them. When actin binding by synthetic peptides corresponding to the first ten residues of rabbit skeletal A1-type ELC was monitored by NMR no significant differences were seen between a peptide with an unmodified N-terminal residue and one with trimethylalanine [25]. However, the addition of three methyl groups to the N-terminus must be an energetically costly exercise for the organism and we would, therefore, expect it to serve some purpose *in vivo*. We suggest two possible

explanations for this apparent paradox. The measurements we make are steady state ones in order to determine the apparent K_M for actin and k_{cat} for MgATP turnover. It may be that the main influence of the trimethylalanine group is on initial pre-steady state steps in the pathway whose influence is not reflected significantly in K_M and k_{cat}. Alternatively it could be that a trimethylalanine blocking group or a free amino terminus contribute approximately equally to binding actin and the function of the trimethylalanine group is to protect the N-terminus of the ELC from degradation within the cell. Trimethylalanine may have been selected over other alternative (less energetically costly) N-terminal modifications (such as acetylation) as it retains a positive charge at the N-terminus which can contribute towards the binding affinity between the ELC and actin. It is interesting to note in this context that A2-like ELCs (which do not interact with actin [14]) are N-terminally blocked with acetyl groups rather than trimethylalanine.

Conclusions.

These results clearly show the importance of two amino acid side chains in the kinetic modulation of myosin by A1-type ELCs. They further show that this system is amenable to this sort of analysis. The kinetic assays not only differentiate between S1(A1)-like and S1(A2)-like kinetics, but can also distinguish more subtle changes resulting from single amino acid substitutions. We have established from these experiments that positive charges at the N-terminus are important in kinetic modulation and our cross-linking results suggest that reducing this charge decreases the affinity of the ELC for actin. This line of investigation would benefit greatly from quantification of the ELC-actin interaction and this should be seen as a priority for future investigations.

We have established in this and previous work the importance of the N-terminal residues of A1-type ELCs in modulating myosin motor function. This modulation results directly from these residues binding to actin and these two events are tightly coupled. What remains to be discovered is how this actin binding event some 8 to 10 nm from the active site of myosin (as judged from the models of acto-S1 derived from

the crystal structures of the proteins [26-28]) results in kinetic modulation. In this context, the results with HmAtELC(K4D) are particularly interesting. Although the light chain cross-links to actin (but to a lesser extent than the other mutants and the wild type), its k_{cat} is essentially S1(A2)-like. This suggests that the free energy of binding resulting from the ELC-actin interaction is used to drive conformational changes which influence the active site. The much weakened interaction in the case of HmAtELC(K4D) is insufficient to drive this process. In the case of HmAtELC(K3A) and HmAtELC(K4A), the binding energy is only sufficient to drive the process part-way. Communication between the light chain binding domain and the motor domain is a common feature of all muscle myosin types; it remains to be seen if the pathways of communication between the light chains and the active site also have features in common.

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Table 1: Actin activated MgATPase kinetic constants for hybrid S1s formed between rabbit skeletal S1 and the various ELC mutants described in this paper.

Values for rabbit skeletal S1(A1), S1(A2) and a hybrid formed with the wild type HmAtELC are given for comparison. The methods for forming the hybrids and determining the kinetic parameters are given in the text. Values are shown plus/minus the standard error. n is the number of separate determinations.

Hybrid	K_M for actin / μM	k_{cat} / s^{-1}	n
S1(A1)	4.2±0.6	13.7±1.1	20
S1(A2)	31.9±9.9	34.6±9.5	24
S1(HmAtELC)	5.4±1.5	15.3±2.8	32
S1(HmAtELC.A1V)	5.2±0.8	15.7±1.5	24
S1(HmAtELC.K4R)	3.5±0.4	14.3±0.9	22
S1(HmAtELC.K4A)	10.8±1.6	20.6±2.3	24
S1(HmAtELC.K4D)	22.8±6.7	28.3±7.0	24
S1(HmAtELC.K3A)	9.4±0.9	15.7±1.1	22

FIGURE LEGENDS

Figure 1: Sequences of the first ten amino acids of the wild type [12] and mutant HmAtELCs used in this study. The sequence of rabbit skeletal A1-type ELC (RbSkA1-ELC) [6] is included for comparison. The dots indicate continuing sequence. A gap has been introduced into the sequence of the rabbit skeletal protein in order to maximise the sequence similarity.

Figure 2: Increasing the size of the side chain at position 1 does not affect the kinetic properties of the resulting hybrid S1 or the actin binding properties of the ELC.
a. Actin activated MgATPase kinetics of S1(HmAtELC.A1V). The line was fitted to the data by non-linear curve fitting using the program Fig-P for Windows.
b. Cross-linking of the HmAtELC(A1V) to F-actin. Lane 1, HmAtELC(A1V) alone; lane 2, wild type HmAtELC plus F-actin; lane 3, HmAtELC(A1V) plus F-actin. The cross-linker EDC was present in all cases as described in experimental procedures. The concentration of all proteins was 20 μM.

Figure 3: The positive charge at positions three and four is important in determining kinetic modulation and actin binding.
a. Actin activated MgATPase kinetics of S1(HmAtELC.K4R) (circles), S1(HmAtELC.K4A) (squares) and S1(HmAtELC.K4D) (triangles).
b. Actin activated MgATPase kinetics of S1(HmAtELC.K4A).
c. Cross-linking of these mutants to F-actin. Lane 1, Wild type HmAtELC; lane 2, HmAtELC plus F-actin; lane 3, HmAtELC(K4R) plus F-actin; lane 4, HmAtELC(K4A) plus F-actin; lane 5, HmAtELC(K4D) plus F-actin; lane 6, HmAtELC(K3A) plus F-actin; lane 7, HmAtELC plus F-actin; lane 8, F-actin. Except in the case of lane 7, EDC was present in all cases. The concentration of all proteins was 20 μM.

Figure 4: The correlation between the k_{cat} for MgATP turnover and the apparent K_M for actin. The values are those shown in table 1 derived by non-linear curve fitting and the error bars are the standard errors obtained in this process. The line is the linear regression line as calculated by Fig-P for Windows.

5.2B ADDITIONAL INFORMATION.

5.2B.1 Testing the requirement for a positive charge at the N-terminus: the HmAtELC(+D0) mutant.

In order to investigate the general requirements for positive charge at the N-terminus of A1-type ELCs, a negatively charged aspartate residue was introduced at the N-terminus (HmAtELC(+D0), having the N-terminal amino acid sequence, M D A P K K P E P K K…) using similar methods to those described in Paper III. Although this mutant was readily expressed, it proved difficult to separate its hybrid from residual S1(A2). The two proteins eluted as a single peak with enrichment of S1(HmAtELC(+D0)) towards the end of this peak. However, although these fractions were enriched in the hybrid, they were by no means pure. It is interesting that a single amino acid addition (representing a net charge change of -1) could have such profound effects on the separation properties of the resultant hybrid. This made any serious kinetic analysis difficult. The following values were obtained: $K_M = 7.2 \pm 0.9$ μM and $k_{cat} = 12.2 \pm 1.0$ s^{-1}. In contrast to all the other hybrids analysed in this manner, examination of the residual plot, following fitting to the equation: activity = (k_{cat} [Actin])/(K_M + [Actin]), was not random (data not shown), suggesting that this simple model does not adequately describe the data. The simplest explanantion for this is that the hybrid was contaminated with significant quantities of S1(A2).

5.2B.2 Variable expression levels of mutant ELCs.

Mutation of codon 4, AAG (Lys) to GAG (Glu) resulted in a construct from which no protein expression could be detected. The HmAtELC(K4D) construct described in paper III did express and could be purified in reasonable yields. It is interesting to note that such small changes in the gene sequence can have such profound effects on the level of expression.

All the other mutants described expressed well - although HmAtELC(A1V) did so at a noticeably lower level than the remainder. This seemingly unpredictable level of

expression is a problem which has been encountered in this laboratory before (Al-Hillawi *et al.* 1994) and for which there seems (unsatisfyingly) to be no solution other than for trial and error.

5.3 DISCUSSION.

Having established the importance of the positively charged side chains at positions 3 and 4 and that the assay system is sufficiently sensitive to detect changes resulting from single amino acid substitutions, there is clearly much scope in this area of work. The work on the lysines 3 and 4 could be extended by making mutations at both sites simultaneously to further assess the relative contributions of both residues.

The biggest drawback to this work is our inability to quantify the strength of actin binding by the wild type and mutant ELCs (see section 3.3). It would be very interesting to see if the binding constants correlated with the observed apparent K_M's for actin. This should, therefore, be a major aim of future work in this area. Another great advantage in making informed choices about which residues to mutate would be a structure of the N-terminal peptide in the conformation it adopts when bound to actin. This could be obtained by application of transferred nuclear Overhauser effect experiments - an established NMR technique for determining the structures of small peptides and other molecules bound to much larger entities (Clore and Gronenborn, 1985). Such a structure might also explain the apparent disagreement between the existing NMR data which suggests that lysine 4 is more important in the ELC-actin interaction than lysine 3 (KJ Smith and HR Trayer, unpublished data) and the mutagenic studies discussed above which suggest that they are both equally important.

Other interesting targets for mutagenesis would be the conserved lysine residues at positions 8 and 9 (HmAtELC sequence). These could be investigated alone and in combination with those at positions 3 and 4. The spacing between these two lysine patches varies between 2 and 3 amino acid residues (see figure 5.1). It would be interesting to see if this spacing has any functional effect by altering its length.

A method for determining the binding constant between the mutant ELCs and actin, further mutagenesis data (especially if the effects of these mutant hybrids were assessed in an *in vitro* motility assay as well as in test-tube kinetic assays), and a structure of the N-terminal residues of the ELC bound to actin should reveal a great deal about the interaction between A1-type ELCs and actin at the molecular level.

Chapter Six:

The Interaction Between *Drosophila* Regulatory Light Chain and Actin. Does it function like an A1-type ELC?

6.1 INTRODUCTION.

6.1.1 Adaptations of muscles to flight: the importance of stretch activation.

When most muscles are subjected to a rapid stretch there is, initially, an increase in tension which then decays rapidly. This decay is followed by a sustained rise in tension. The effect is most significant in vertebrate cardiac muscles and insect flight muscles. Biochemically this phenomenon of stretch activation can be explained by assuming that the initial resistance results from myosin heads which are associated with actin filament opposing the length increase until the applied force overcomes these interactions and tension drops rapidly. When the length is no longer being changed myosin can reassociate with actin and tension rises once again.

The existence of this effect means that muscles can, in effect, activate one another if opposing pairs stretch each other alternately. This sets up an oscillatory motion (of the type seen in the heart and in insect flight muscles) which can be sustained with minimal energy input as only the initial contraction needs to be stimulated by calcium ions and the oscillatory contractions are sustained by stretch activation - thus saving energy on ion pumping.

The insect body is adapted to take advantage of this effect. The inertia of the wings and elasticity of the thorax set the resonant frequency of the system and the indirect flight muscles (IFM) contract at this frequency in opposing pairs, powering the wing beats and stretch activating the other IFM of the pair (see review by Sparrow, 1995).

6.1.2 The *Drosophila* Regulatory Light Chain.

Two dimensional gel electrophoresis shows a large number (>10) of different myosin light chains in *Drosophila* muscle (Takano-Ohmuro *et al.* 1983). Some of these are developmentally regulated being found predominantly in either the embryo or adult fly and some are phosphorylated forms of others (Takano-Ohmuro *et al.* 1990). These workers identified two forms of RLC (and phosphorylated forms of each) by their

ability to be phosphorylated by *Drosophila* myosin light chain kinase. Interestingly, only one gene (and one mRNA transcript) for the RLC can be isolated (Parker *et al.* 1985; Toffenetti *et al.* 1987) suggesting that the different proteins observed result from post-translational modifications of the same product rather than from the translation of more than one mRNA transcript. The predicted protein sequence shows similarity to vertebrate RLCs except for an additional 55 to 60 amino acid residues at the N-terminus of the *Drosophila* sequence (Parker *et al.* 1985; Toffenetti *et al.* 1987) (see below). The positions of the introns in the *Drosophila* RLC gene correspond to those in the rat gene - suggesting that the two sequences are related evolutionarily (Parker *et al.* 1985). The protein product is N-terminally blocked *in vivo* and when produced *in vitro* in a rabbit reticulocyte lysate. The protein produced in the reticulocyte lysate is acetylated (blocking acetylation in the *in vitro* system results in an unblocked translation product) (Toffenetti *et al.* 1987).

The RLC is required for normal muscle development and for the viability of the fly. Flies homozygous for a truncated form of the gene coding for the RLC (MLC-2^{E38}) are not viable and heterozygotes have aberrant muscle structure, poor flight and reduced wing beat frequencies (Warmke *et al.* 1992).

6.1.3 Functional significance of phosphorylation of the *Drosophila* RLC.

Like its vertebrate homologue, the *Drosophila* RLC can be phosphorylated by myosin light chain kinase (Takano-Ohmuro *et al.* 1990). This phosphorylation results in an increase (approximately two-fold) in the actin activated MgATPase activity of myosin (Takahashi *et al.* 1990a). In a flightless *Drosophila* mutant (mfd^{-}) no phosphorylated RLCs can be detected on two dimensional gel electrophoresis and the actin activated MgATPase activity is constituitively low. This suggests that phosphorylation of the RLC is important in switching on the IFM prior to flight (Takahashi *et al.* 1990b).

Comparison of the protein sequence of the *Drosophila* RLC to other RLC sequences shows that serines 66 and 67 are likely to be the sites of phosphorylation by myosin light chain kinase. Indeed these sites are phosphorylated by the kinase *in vitro* (Tohtong

et al. 1995). Complementation of MLC-2^{E38} homozygotes with transfected copies of the wild type gene rescues this phenotype (Warmke *et al.* 1992) and this provides a system into which mutant RLCs can be introduced and their effects assayed *in vivo*. Mutation of serines 66 and 67 to alanine results in a flightless phenotype, with low wing beat frequency but normal muscle ultrastructure. Of these two phosphorylation sites, serine 66 is more important that serine 67; the S66A mutant results in much greater phenotypic effects than S67A. Muscle fibres prepared from the indirect flight muscles containing only RLC(S66A,S67A) show large reductions in their stretch activation response with no corresponding reduction in isometric tension (Tohtong *et al.* 1995). These authors suggest that the link between phosphorylation of the RLC and increased responsiveness to stretch activation is that the phosphorylated RLC provides an additional interaction between myosin and actin. They propose a model in which the additional amino acids seen at the N-terminus of the *Drosophila* RLC are folded (perhaps such that it interacts with the main body of the RLC at, or near, the phosphorylation site) in the dephosphorylated state becoming more extended on phosphorylation in order to reach across and contact the actin filament (figure 6.1).

6.1.4 Does the *Drosophila* RLC have a similar function to A1-type ELCs from vertebrate systems?

Figure 6.2 shows an alignment of the *Drosophila* RLC sequence with other light chain sequences. It is immediately apparent that while the C-terminal 190 residues of the *Drosophila* RLC show greatest similarity with rabbit skeletal RLC, the N-terminal region (which has no corresponding residues in the rabbit RLC) shows some similarity to the N-terminal extensions of A1-type ELCs. In particular, the two classes of light chain show similarity at the extreme N-terminus (ie the actin binding site of A1-type ELCs) and in the proline rich region C-terminal to this. That the proline rich region of the *Drosophila* RLC is an extended structure is suggested by the fact that, like A1-type ELCs, it migrates on SDS-PAGE at an apparent molecular mass higher than its predicted molecular mass (30 kDa as opposed to 24 kDa) (Toffenetti *et al.* 1987). If the N-terminus of the *Drosophila* RLC does act as an actin binding site this would provide

the additional link between myosin and actin postulated by Tohtong and co-workers (Tohtong *et al.* 1995).

The actin binding properties of the *Drosophila* RLC were investigated by applying the cross-linking methodology that was developed for the HmAtELC. Furthermore, the location of the binding site could be narrowed down by using a peptide corresponding to the RLC N-terminal sequence. The success of these experiments provides the basis for re-evaluation of the Tohtong model.

6.2 BINDING OF THE *DROSOPHILA* RLC TO F-ACTIN.

6.2.1 Intact *Drosophila* RLC.

Recombinant *Drosophila* RLC was supplied as a freeze dried powder by our collaborator Dr David Maughan (University of Vermont, USA). The protein was reconstituted by dissolving in 5 mM TEA.HCl, pH 7.5, 0.25 mM DTT in the presence of 1 mg DTT per mg freeze dried powder and then dialysed against this buffer overnight. When *Drosophila* RLC and F-actin were exposed to the zero-length cross-linker EDC using the method described for the HmAtELC (Section 2C.7.1 and Paper I) a band corresponding to an RLC-actin product (figure 6.3) is seen on SDS-PAGE analysis. This band does not appear when the RLC alone is exposed to the cross-linker, confirming the assignment of this band. The RLC-actin product shows a similar dependence on ionic strength to the wild type HmAtELC (see section 3.2B.4) (data not shown). This experiment shows that the *Drosophila* RLC interacts with F-actin, but does not give us any information about the site of interaction.

6.2.2 *Drosophila* 1-21 peptide.

In order to localise the actin binding site in the *Drosophila* RLC, a peptide corresponding to the region of the RLC showing similarity to the actin binding site of

A1-type ELCs was synthesised (residues 1 to 21). This peptide could be cross-linked to F-actin (figure 6.4) using a protocol identical to that used to cross-link the rabbit skeletal 1-32 peptide (section 3.2B.3). This narrows down the site of interaction to these first twenty residues and provides compelling, if circumstantial, evidence that the residues that show such a high degree of similarity to A1-type ELCs (ie ...A-D-E-K-K-K-V-K-K...) also encode an actin binding site.

6.3 DISCUSSION.

Although the direct interaction between A1-type ELCs and F-actin has long been predicted (Trayer *et al.* 1987; Milligan *et al.* 1990; Labbé *et al.* 1986; Winstanley *et al.* 1977), this is the first evidence for a direct RLC-actin contact. Given the similarity of the N-terminal sequences of the *Drosophila* RLC and A1-type ELCs (both have a lysine rich sequence at the immediate N-terminus with a proline rich sequence C-terminal to it) it is tempting to hypothesise that the *Drosophila* RLC fulfils a similar rôle to the A1-type ELC. Since we only have the free light chain available to us, it is not possible to assess the kinetic effects of this RLC (compared say to a deletion mutant lacking residues 1-20) in any form of reconstituted system. This problem might be addressed by expressing a chimeric light chain consisting of the HmAtELC with the first eleven residues substituted for the *Drosophila* RLC sequence. Hybridising this light chain into rabbit skeletal S1(A2) and carrying out kinetic assays of the type described for the HmAtELC (Chapters 3, 4, and 5) would enable us to determine if the N-terminal sequence of the *Drosophila* RLC is capable of modulating myosin motor function. The actin binding site could be located more precisely by using deletion mutagenesis in a similar manner to that described in Chapter 3 for the HmAtELC. If the chimeric system described above proved a success then it could be used to assess the rôle of individual amino acids in the actin binding site by site directed mutagenesis in a similar manner to that described in Chapter 5. Like the work with the HmAtELC, this work would benefit greatly from a quantitative understanding: any method for measuring the binding constant between the HmAtELC and actin is likely to be applicable to this system.

These results give some support for the model advanced by Tohtong and co-workers (Tohtong *et al.* 1995). This model requires a direct interaction between the *Drosophila* RLC and actin and evidence of this has been provided. However, their model suggests that only phosphorylated RLC should interact with actin since they postulate that the actin binding site folds back to interact with the phosphorylation site in the dephosphorylated state. The protein used in this experiment, being bacterially expressed, would be totally dephosphorylated and yet clearly binds. There are a number of possible explanations for this contradiction. If the equilibrium constant between the folded-back and extended forms of the dephosphorylated protein is such that a small amount of the extended form exists at any one time, then this could bind actin transiently and this could be captured by the cross-linker (figure 6.5a).

Alternatively, it is not necessary to postulate that the N-terminus of the RLC interacts with the phosphorylation site in the dephosphorylated protein in order to explain why it does not bind actin in this state. Phosphorylation might result in a conformational change in the N-terminal segment which has the effect of moving the actin binding site closer to actin. Indeed if the proline-rich region functions as a rigid, extended spacer arm like the similar sequence in A1-type ELCs it is difficult to see how this region could fold back on itself; the energetic arguments developed in the Discussion section of Paper II would also apply. If phosphorylation results in a change of orientation of the arm then this would only alter the RLC's ability to bind actin when its position is constrained by the heavy chain. In the free RLC, the actin binding site would be exposed (regardless of the phosphorylation state) and available for interaction thus explaining the cross-linking data (figure 6.5b). Indeed conformational changes resulting from RLC phosphorylation might not even involve the N-terminus of the light chain. The effect might be to alter the relative positions of myosin and actin, moving the actin binding site closer to the surface of actin (figure 6.5c). Such a movement of myosin and actin has been observed on the phosphorylation of the RLC in striated muscle (Sweeney *et al.* 1993).

Of course there is no direct evidence that phosphorylation of the *Drosophila* RLC results in increased RLC-actin affinity (or indeed that it controls this interaction in any

way). This assumption results from the idea that the RLC-actin interaction promotes the stretch activation response (Tohtong *et al.* 1995) and this may well be so. However, the data from vertebrate striated muscle (Lowey *et al.* 1993b; Wagner and Weeds, 1977; Bottinelli *et al.* 1994a) and Chapter 3) suggest that A1-type ELCs result in *slower* muscle phenotypes from which it might be inferred that the *Drosophila* RLC-actin interaction slows down *Drosophila* myosin. Flight may well require a faster myosin isoenzyme and therefore it is possible that the effect of phosphorylation is to disrupt the RLC-actin interaction, resulting in (effectively) an instant isoenzyme transition from S1(A1)-like to S1(A2)-like.

These hypotheses can only be addressed by isolating whole *Drosophila* myosin (or some defined fragment of it that retains the RLC) in both fully phosphorylated and fully dephosphorylated forms and monitoring the RLC-actin interaction by cross-linking or some other more sophisticated method. Understanding the mechanisms of stretch activated contraction in the IFM of *Drosophila*, and in particular the rôle of the RLC in this process, may well shed light on the analogous problem in cardiac muscle. Indeed if the light chain does play a part in the stretch activation mechanism, then this may explain why A1-type ELCs are the only ELC isoform found in cardiac muscle.

Chapter Seven:

General Discussion.

Most of the work in this thesis has been presented in the form of papers and the results described in them discussed fully in the appropriate sections of the papers. Rather than recapitulate this, this chapter focuses mainly on some of the unanswered questions in this field and suggests ways of addressing them.

7.1 Advantages of a Recombinant Source of HmAtELC.

The HmAtELC can be readily expressed in, and purified from, *E. coli* (Chapter 3). This opened up the possibility of testing hypotheses about the function of A1-type ELCs by making defined changes to the protein (Chapters 3, 4 and 5). Some of the potential for further experiments of this nature has been addressed in the discussion sections of these chapters and the papers contained therein. The possession of a recombinant source of HmAtELC allows further possibilities. The protein can be purified in the absence of urea (in contrast to purification of ELCs from skeletal muscle, where strong denaturants are required to dissociate the ELC from the heavy chain (Trayer and Trayer, 1988)). That the native protein behaves in a similar manner to those that have been renatured from urea (both in its actin binding properties and its effect on the actin activated MgATPase of S1 - Chapter 3) gives us added confidence that previous work with renatured ELCs (eg Trayer and Trayer, 1988; Wagner and Weeds, 1977; Henry *et al.* 1985b) is valid.

By working with recombinant proteins, it is possible to work with a human ELC. Along with cd TnT and cd TnI, the atrial ELC is released into the general circulation after major damage to the heart such as heart attacks, myocardial infarction and transplantation (Uchino *et al.* 1994; Mair *et al.* 1994). The presence of the HmAtELC in the bloodstream is an indicator that these events have occurred and measuring the time-course of the release, build up and decrease in the amount of protein, the course of a patient's condition and recovery can be monitored. The potential exists, therefore, to use the recombinant HmAtELC to develop monoclonal antibodies specific to the HmAtELC for use in diagnostic kits. The recombinant protein would also provide standards for any such kit. The HmAtELC is an important marker for these conditions as it persists longest (Uchino *et al.* 1994).

Access to recombinant protein makes any structural or dynamic study by NMR more feasible. Not only can large quantities of protein be produced with relative ease and speed, the protein can be labelled with NMR active nuclei, such as ^{13}C and ^{15}N which should make any detailed assignment easier. Furthermore, specific regions of the protein could be expressed and these regions investigated if working with the whole protein proved problematic. This might well prove to be the case given the low solubility of the ELC and its tendency to aggregate in solution. The latter problem might, in part, be relieved by mutating the two cysteine residues (Cys 77 and Cys 183) to serine - another advantage of working with the recombinant protein.

7.2 How Do A1-type ELCs Work?

This study has established, unequivocally, that actin binding by the N-terminus of A1-type ELCs results directly in kinetic modulation of the myosin motor. A (much more difficult) question which remains unanswered is how this actin binding event exerts its influence on the active site of the enzyme some 8 to 10 nm away. The effects of A1-type ELCs that have been observed are on the steady state kinetic parameters (Weeds and Taylor, 1975; Wagner and Weeds, 1977 and Chapters 3,4 and 5). It is quite possible that there are other effects too. Furthermore, the observed changes in apparent K_M and k_{cat} must result from changes at one, or more, fundamental points in the MgATPase cycle (section 1.1.7). It is also important to ask how information is transmitted within the acto-S1 complex to bring about these changes. This leads to the more general question of how an actin binding event far from the active site can bring about such changes. It is also important to consider if the ELC-actin interaction might influence any properties of the muscle fibre, other than the actin activated MgATPase activity of myosin.

7.2.1 Which stage(s) of the MgATPase cycle do A1-type ELCs influence?

In the absence of any non-steady state MgATPase kinetic data it is impossible to answer this question unequivocally. This question can be, effectively, re-phrased as: which rate constant(s) is(are) different with S1(A1) compared to S1(A2)? Since the presence of A1-type light chains decreases the k_{cat} of S1 (Weeds and Taylor, 1975; Wagner and Weeds, 1977; Wagner *et al.* 1979 and Paper I), this suggests that the rate determining step is affected (since k_{cat} is, in effect a measure of the rate constant of the rate determining step, even if what is measured chemically is the product of a different step). Unfortunately, there is no agreement as to the nature of the rate determining step. The model of Eisenberg and Greene (Eisenberg and Greene, 1980) defines the rate determining step as being an isomerisation of the (weak binding) acto-myosin-MgADP-P_i complex to a state that is capable of releasing P_i. Others assign it to be the release of P_i itself and the nature of the step may well depend on whether, or not, actin is present. Either way, it is clear from the crystal structure (Rayment *et al.* 1993b) that the extreme N-terminus of the ELC cannot, directly influence the ADP-P_i binding site. It seems likely that subtle changes in the acto-S1(A1) complex initiated by the ELC-actin interaction, occurring prior to the rate determining step, influence the rate constant of this step.

The apparent K_M for actin reflects the strength of interaction between actin and S1. However, it is not clear from our experiments which stage (or stages) is most affected. Structural interpretations of the MgATPase cycle suggest that the weak to strong binding transition results in the involvement of more binding sites on S1 for actin (Rayment *et al.* 1993a; Rayment and Holden, 1994; Cooke, 1993; Spudich, 1994). If this is the case then it might be assumed that the A1-type ELC-actin interactions play a part in the strong (ie MgADP bound) state - this influencing the transition from strained (post P_i release) 45° state of the Eisenberg and Greene model to the non-strained state.

7.2.2 Pathways of communication in the acto-S1 complex.

Since the actin-ELC interaction does not, directly, influence the ATPase site of myosin, information must be transmitted through the acto-S1 complex - presumably as a series of co-operative changes within the complex. Without even knowledge of the structures of the main stages of the MgATPase cycle, it is impossible to say anything about the nature of these changes in detail. However, we can pose one fundamental question: is information transmitted through the actin filament or through S1 (figure 7.1). The results with the HmAtELC2XP mutant hybrid (which binds actin but does not modulate the MgATPase kinetics) provides some evidence that the through actin pathway is the predominant route (see Paper II).

However, before this can be answered unequivocally, many other questions need to be addressed. The most immediate is to confirm the assumption that, in S1(HmAtELC2XP), the light chain binds to a more distant actin than the light chain in S1(HmAtELC) (Figure 7.2a). This could be confirmed by comparing the cross-linking of S1(HmAtELC2XP) and S1(HmAtELC) to cross-linked actin dimers, in a manner analogous to that described in Paper I for the wild type ELC and G-actin-DNase-I (figure 7.2b). Cross-linked actin dimers could be prepared with relative ease: they appear as a by-product in the glutaraldehyde cross-linking of F-actin; they could be separated from cross-linked F-actin by spinning the polymer down in buffer A (see section 2C.9) and from the monomer (and larger oligomers) by gel filtration chromatography. The dimers could be "capped" with DNase-I to prevent any filament formation and eliminate any interference from cross-filament dimers.

For this and other work it is also important to locate the site of interaction on actin more precisely. NMR (Trayer *et al.* 1987) and electron microscopy image reconstruction (Milligan *et al.* 1990) experiments suggest that it lies towards the C-terminus of actin, in sub-domain 1. Chemical or enzymatic digestion of the HmAtELC-actin cross-linked product might provide the key to answering this question. (Use of the HmAtELC(K4R) mutant in these experiments might be preferable, since this mutant gives a higher yield of adduct, Paper III). Comparison of SDS-PAGE profiles of cross-linked and non-cross-linked complexes after digestion should reveal which peptide fragments are involved in the reaction (figure 7.3). This product could then be subjected to amino

acid sequencing. Residues involved in the cross-linking reaction would not be detected and would appear as gaps in the sequence (this strategy has been applied recently to the bacterial chaperonin GroEL (Mayhew *et al.* 1996)). (Only actin sequence should be observed as the N-terminal residue of the recombinant HmAtELC is involved in the cross-linking, Paper I).

Once the binding site has been located, this would enable the use of peptides encoding the binding sites to perturb potential pathways within acto-S1. If such experiments proved successful - and care should be taken in their interpretation as peptides can cause non-specific effects - they should distinguish between the two pathways. In the presence of their complementary site, the peptides should adopt a conformation similar to that seen in the intact protein - and mimic the effect of the binding event. Thus in acto-S1(A1) a peptide corresponding to the N-terminal residues of A1-type ELCs should compete with A1 for its site on actin (figure 7.4a). If the actin pathway predominates, then little or no effect should be observed since the pathway is initiated by occupation of the site on actin: it cannot distinguish whether this is filled by an ELC or a peptide mimic. However, displacement of the ELC from actin by this peptide would disrupt the through S1 pathway. Thus, increasing concentrations of the peptide would result in a transformation from wild type (S1(A1)-like) kinetics to S1(A2)-like kinetics. If this pathway operates, similar effects would be seen in S1(A2). The peptide could substitute for A1-type ELCs in the through actin pathway, but not in the through-S1 pathway as there would be no link between the peptide and the ELC.

The actin peptide would have almost reverse effects. In S1(A1), it would bind the N-terminus of the ELC and initiate the through-S1 pathway with equal efficiency to whole actin. (This assumes the ELC binds to a single site on actin, coded for by a continuous sequence of polypeptide, not residues from different parts of the sequence which happen to be close in space). However increasing concentrations of the peptide would disrupt the through-actin pathway - preventing the ELC from binding actin and triggering the process (figure 7.4b).

Although the ELC isoform does not affect the non-actin activated MgATPase kinetics of S1 (Weeds and Taylor, 1975), if the through S1 pathway operates, then the addition of actin peptide to S1(A1) in the absence of actin might affect the rate of MgATP turnover (figure 7.4c). If it did not then it would suggest that the pathway requires a "gate" to be opened by the binding of the heavy chain to actin before it can transmit information to the active site

One attempt to perform experiments of this nature has been reported (Hayashibara and Miyanishi, 1994). However, this work can be criticised for the small number of data points and the way in which the data were analysed (see section 1.4.7). What is required to answer these questions is quantitative information about how K_M and k_{cat} depend on the peptide:S1 molar ratio. If these experiments behave as described above then these should either be unaffected or show a saturatable transition from values characteristic of one isoform to the other depending on which peptide is used and which pathway predominates. If the prediction made in Chapter 5 that the apparent K_M controls k_{cat} is correct then we would expect the effects on one parameter to closely mirror the effects on the other. The peptide:S1 ratio required to give half the effect will be a measure of the relative affinity of the intact ELC (in S1) and the peptide (free in solution) for actin - and, therefore, a measure of the contribution of the rigid spacer arm (see discussion section of Paper II) to the affinity of binding.

If the through actin pathway predominates (as the results of chapter 4 suggest), then proteins such as tropomyosin which enhance the co-operativity of the actin filament (Geeves and Lehrer, 1994) might affect the kinetics of S1(HmAtELC2XP). Specifically, we might expect that, in the presence of tropomyosin, coupling between actin binding (reported by K_M) and kinetic modulation (k_{cat}) would be (at least partially) restored. Thus it is necessary to determine the actin activated MgATPase kinetics of S1(HmAtELC), S1(HmAtELCΔ(1-45)) and S1(HmAtELC2XP) using actin-tropomyosin.

The sort of experiments outlined in this section should enable the contributions of each pathway to be assessed. The results may well have important implications not only for

this system but also for thick filament regulated systems where messages also pass from the light chain binding domain to the ATPase site. The table below summarises the various experiments and the expected outcomes for each pathway.

Experiment	Actin pathway	S1 pathway
Actin peptide + S1(A1)	Transition to S1(A2)-like kinetics	No effect (possible effect in the absence of actin)
ELC peptide + S1(A1)	No effect	Transition to S1(A2)-like kinetics
ELC peptide + S1(A2)	Transition to S1(A1)-like kinetics	No effect
Addition of tropomyosin to the assay system	(Partial) restoration of coupling between actin binding and kinetic modulation in S1(HmAtELC2XP)	No effect (?)

7.2.3 How might A1-type ELCs affect the MgATPase cycle?

7.2.3.1 Potential effects on F-actin.

If information is transmitted *via* the actin filament then what kind of changes might we expect? The effect of A1-type ELCs is to reduce k_{cat} (Wagner and Weeds, 1977 and Chapter 3) resulting in a "slower" myosin motor. Thus, A1-type ELCs could be seen as partial inhibitors of the MgATPase activity of S1. TnI also acts as an inhibitor of this activity - although it is more potent resulting in an almost total inhibition at a TnI:S1 molar ratio of 5:1 (Al-Hillawi *et al.* 1994). Potentially A1-type ELCs and TnI might act by similar mechanisms, with A1-type ELCs (partially) mimicking the binding of TnI to actin. If this is so then we would expect them to have overlapping binding sites. This question was addressed by attempting to form a TnI-actin-ELC ternary complex: if such a complex can be formed then this conjecture is unlikely to be true. Unfortunately

these experiments gave ambiguous results and, therefore, this is an area requiring further study. Alternative cross-linkers could be used and if methods to quantify the ELC-actin interaction (such as fluorescence or improved BIAcore technology) are developed then the effect of TnI as a competitor of the interaction could be monitored directly.

7.2.3.2 Potential effects on S1

Recently, the various modes of binding of calmodulin and related proteins (including myosin light chains) to their target peptides have been analysed. Three conformations can be distinguished: open, semi-open and closed (Houdusse and Cohen, 1995; Ikura, 1996; Swindells and Ikura, 1996). The calcium bound, triggering conformations of calmodulin and the C-terminal sites of TnC are open conformations. In this conformation, the EF-hands form a V-shaped groove into which α-helical peptides can fit. In the closed conformation (eg the calcium-free state of calmodulin), this groove is not present and target binding in this manner is not possible. The C-terminal half of the ELC in both scallop and chicken myosin binds in a semi-open conformation (figure 7.5a) as does the C-terminus of the RLC (Houdusse and Cohen, 1995). The N-terminus of the ELC is in the closed conformation and makes few contacts with the heavy chain, involving only residues on the exposed surface of this domain. Figure 7.5b shows a schematic representation of the ELC-heavy chain interactions.

The analysis of different modes of binding of calmodulin-like proteins to their target sequences and the identification of the contacts between light chains and the IQ motifs they bind (Houdusse and Cohen, 1995; Houdusse and Cohen, 1996; Swindells and Ikura, 1996) highlight those residues which may be involved in information transmission between the ELC and the heavy chain. Mutagenesis of these residues would serve two purposes: it would enable the requirements for molecular recognition between the heavy chain and the ELC to be investigated (one very interesting question is how similar IQ motifs discriminate between the ELC, the RLC and, indeed calmodulin) and it would, potentially, allow disruption of any through S1 pathway of information transmission. If such changes are possible then the expected phenotype

would be an ELC which confers more S1(A2)-like kinetics on S1 than the wild type but which binds actin to the same extent (both free and bound to the heavy chain of S1).

7.2.4 What other effects could A1-type ELCs have?

So far, only the effects of A1-type ELCs on the steady state solution MgATPase kinetics have been discussed. In muscle tissue, the constrained arrangement of the actin and myosin filaments mean that mechanical effects are also important. The importance of stretch activation in *Drosophila* muscle and how this may be mediated by the RLC has been discussed in chapter 6. It is possible that this effect (mediated in this case by the ELC) is also significant in vertebrate cardiac muscle.

One of the most exciting discoveries in light chain research in the last year was the isolation of a genetic disease occurring at a locus that maps to the human ventricular ELC. Although cardiomyopathies have been reported which map to the myosin heavy chain, C-protein, tropomyosin and cd TnT (Marian and Roberts, 1995; Davies and Mckenna, 1995), this is the first report of an ELC abnormality. The two mutations (M149V and R154H) occur in residues which are conserved through ELC types (see figure 5.1) (Poetter *et al.* 1996). Individuals carrying these mutations have abnormal cardiac muscle structure and heart function. Mutations in the heavy chain associated with familial hypertrophic cardiomyopathy (many of which occur in residues forming part of the ATP and actin binding sites (Rayment *et al.* 1995)) result in decreased sliding velocity in *in vitro* motility assays . This is in marked contrast to the M149V ELC mutation which results in an increased sliding velocity (the R154H mutation was not assayed) (Poetter *et al.* 1996). These authors suggest that this mutation may affect the stretch activation response of ventricular muscle, by interfering with the elastic properties of the light chain binding domain. Another possibility is that this residue is involved in the through S1 pathway of information transmission from the ELC to the active site. It would, therefore be interesting to make the equivalent mutations in the HmAtELC (M150V, R155H) and assess their effects on the actin activated MgATPase activity of S1. Such experiments would complement the skinned fibre experiments planned by Poetter et al.

In general, that the N-terminus of the ELC (or RLC in *Drosophila*) can provide an additional point of contact between the actin and myosin filaments means that those containing A1-type ELCs should be more resistant to longitudinal deformation than those containing mainly A2-type ELCs. The magnitude and significance of this effect will depend on whether A1-type ELCs remain attached through most, or only part, of the MgATPase cycle (see section 7.2.1).

7.3 How Appropriate is Surface Plasmon Resonance for the Study of Protein-Protein Interactions?

The proponents of surface plasmon resonance, and the BIAcore system in particular, make substantial claims about its applicability to a wide range of macromolecular interactions (O'Shannessy *et al.* 1994; Raghavan and Bjorkman, 1995; Fisher and Fivash, 1994; Szabo *et al.* 1995). Indeed the system has been used with considerable success in the study of antigen-antibody (Kelley and O'Connell, 1993; Altschuh *et al.* 1992), cell surface receptor-ligand (Brigham-Burke *et al.* 1992; Ward *et al.* 1995; Munir Alam *et al.* 1996) protein-peptide (Ramsdale *et al.* 1993; Takano *et al.* 1994; Wohlhueter *et al.* 1994), and protein-DNA (Bondeson *et al.* 1993; Parsons *et al.* 1995) interactions. However, other than antibody and receptor studies very little in the way of successful protein-protein interaction work has been reported. Much of the work that has may not stand up to close scrutiny. A recent report (Munir Alam *et al.* 1996) of the interaction between the soluble domain of T-cell antigen receptor and its peptide ligand shows residual plots after non-linear curve fitting to a mono-exponential model which are clearly non-random. Most authors do not report residual plots.

In the case of the cd TnC / cd TnI interaction (section 3.3.2.1) the data produced did not stand up to rigorous analysis and in the case of the ELC-actin interaction, no data could be produced due to problems with the immobilisation of actin (section 3.3.2.2). The problems described with these systems result from the negatively charged nature and branched nature of the CM dextran matrix which means that repulsion and mass transport dominate these interactions. This explanation was confirmed by work carried

out with another commercially available surface plasmon resonance based biosensor, IAsys (Affinity Sensors). Two types of sensor chip are available for use in this machine: a CM dextran chip similar to that available for the BIAcore and a flat, amino-silane surface. (Edwards *et al.* 1995) compared the association and dissociation phase kinetics of antigen-antibody interactions on both surfaces. The CM dextran surface gave data that could not be adequately described by a single set of rate constants (residuals were large and non-random when fitted to this model and the derived association rate constant was concentration dependent *cf* cd TnI / cd TnC) but could be fitted to a biexponential model. This kinetic behaviour results not from complexities in the system but from the nature of the resin: analysis of the same systems on the amino-silane surface gave data that could be described by a monoexponential model. Similar effects have also been observed with other antigen-antibody complexes (George *et al.* 1995).

This work on the IAsys system, strongly suggests that the solution to the problems described in section 3.3.2 lies in the development of new sensor chip surfaces (and appropriate immobilisation chemistries) rather than the adoption of ever more complicated immobilisation strategies with the existing surfaces.

Most methods of detecting protein-protein interactions rely on modification of at least one partner (eg the introduction of a fluorescent label) and most mathematical models describing these interactions are, at best, simplifications (Phizicky and Fields, 1995). However, such models must be capable of providing new biological insight, rather than a catalogue of rate constants that cannot be assigned to specific events. Furthermore, if more complex models are used then we must be certain that this complexity exists *in vivo* and has not been introduced by the methodology used to investigate the system.

In conclusion, surface plasmon resonance offers the opportunity to quantify biological interactions with minimal modification of the binding partners and to dissect these interactions to the level of association and dissociation rate constants. However, in order to be as widely applicable to the study of protein-protein interactions as the

manufacturers claim then surfaces which do not interact significantly with the proteins under investigation will need to be developed.

7.4 THE LIMITS OF KINETIC ANALYSIS.

In contrast to enzymatic reactions occurring in the cytoplasm, muscle contraction does not occur in solution. Indeed the Eisenberg and Greene model for the kinetic cycle (Eisenberg and Greene, 1980) *requires* the myosin head and its binding sites on actin to be tethered into a constrained, filamentous network, for the development of force. Furthermore, almost all the work described in this thesis has been carried out *in vitro* with S1. Many *in vivo* situations can be modelled by using S1, actin and other muscle proteins in solution but some cannot. Enzyme assays cannot be used to investigate phenomena such as stretch activation or force generation - both of which could be important in a complete explanation of the rôle of the ELC.

To this end an *in vitro* motility assay system was developed. Although these experiments were not successful in differentiating between S1(A1) and S1(A2), it seems likely that by altering the conditions or methodology of the assay (as discussed in section 3.4) a suitable system will be forthcoming. Once established, the assay system could be used with each mutant to confirm many of the observations of chapters 3,4 and 5. We would expect the sliding velocity to be correlated to the apparent K_M for actin for the site directed mutants. A more interesting case is that of S1(HmAtELC2XP) where it will be important to test whether modulation of the sliding velocity is (like the maximal MgATP turnover rate) uncoupled from actin binding by the ELC.

Beyond this, *in vitro* systems could be used to measure the force developed by the different isoenzymes and the various mutants. Although S1(A1) moves actin more slowly, it might be expected to develop more force (see section 7.2.4). A different kind of assay system (one in which the molecules can be coupled to a sensitive force detector such as an optical trap) is required to measure this and once again conditions under which S1(A1) and S1(A2) give reproducibly different results will have to be developed before the various mutant hybrids can be analysed.

Skinned muscle fibres are an intact system that is amenable to experimental manipulation. Studies using peptides diffused into skinned muscle fibres have been initiated in collaboration with Dr David Maughan (University of Vermont, USA) to study the effect of the N-termini of the HmAtELC and the *Drosophila* RLC on the stretch activation response. As yet this work has yielded no positive results (D Maughan, personal communication). Such work is experimentally difficult and requires the use of appropriate controls. That it has not yet been successful calls the work of (Morano *et al.* 1995) further into doubt.

Ultimately it would be desirable to test each mutant ELC *in vivo*. Although methods for exchanging the ELC in intact muscle fibres have been reported (Sweeney, 1995), these results have yet to be confirmed. The alternative approach of expressing mutant ELCs in transgenic animals, while giving us the opportunity to assess the phenotypic outcome on the whole organism, would have to wait until we have a fuller understanding of ELC expression *in vivo* - in particular how this is co-ordinated with heavy chain gene expression and how the hexameric myosin molecule is assembled in the cell.

7.5 Fine Tuning the Motor.

The main findings of this thesis are summarised below and the principal data collected together in the table on the following page.

1. A1-type myosin essential light chains modulate the solution MgATPase kinetics of myosin subfragment 1. They do this by binding to actin. The binding site is coded for the first eleven (or fewer) amino acid residues of the ELC. In the wild type, actin binding and kinetic modulation are tightly coupled events.
2. The ELC-actin interaction involves a different actin monomer to the one involved in the main myosin heavy chain contacts.
3. The proline-rich region, C-terminal to the actin binding site of the ELC acts as a rigid spacer arm, responsible for correctly positioning the binding site on the surface of actin.

4. Doubling the length of this proline-rich region uncouples actin binding from modulation of the maximum MgATP turnover rate (k_{cat}). This suggests that information is transferred between the actin binding site and the active site of S1 mainly through the actin filament.

5. The size of the side chain at position one is unimportant in determining actin binding and kinetic modulation. A positive charge at positions 3 and 4 is required both for full actin binding and full kinetic modulation. The apparent K_M for actin controls k_{cat} up to a limit set by S1(A2).

6. The regulatory light chain from *Drosophilia* intermediate flight muscle binds actin and may fulfil a similar rôle in this model as the A1-type ELC does in vertebrate striated muscle.

7. Surface plasmon resonance is not an appropriate technique for quantifying the ELC-actin interaction until alternative sensor chip surfaces become available for the BIAcore 2000.

8. Although both S1(A1) and S1(A2) move actin in an *in vitro* motility assay, conditions which adequately distinguished the two isoenzymes were not found. However, it should be possible by further manipulation of the experimental conditions to develop an assay system which does.

ELC	K_M/μM [(a)]	k_{cat}/s^{-1} [(a)]	Kinetic phenotype [(a)]	Cross-linking - free light chain [(b)]	Cross-linking - light chain in S1
RbSkA1	4.2±0.6	13.7±1.1	S1(A1)-like	✓✓✓	Yes
RbSkA2	31.9±9.9	34.6±9.5	S1(A2)-like	Not determined [(c)]	No
HmAtELC	5.4±1.5	15.3±2.8	S1(A1)-like	✓✓✓	Yes
HmAtELCΔ(1-45)	38.9±16.7	43.0±16.8	S1(A2)-like	×	No
HmAtELCΔ(1-11)	20.8±6.9	27.3±7.7	S1(A2)-like	×	No
HmAtELCΔXP	26.6±4.8	24.8±3.8	S1(A2)-like	✓✓✓	No
HmAtELC2XP	5.7±0.7	28.3±2.7	Mixed [(d)]	✓✓✓	Yes
HmAtELC(A1V)	5.2±0.8	15.7±1.5	S1(A1)-like	✓✓✓	Yes
HmAtELC(K4R)	3.5±0.4	14.3±0.9	S1(A1)-like	✓✓✓✓	Yes
HmAtELC(K4A)	10.8±1.6	20.6±2.3	Intermediate	✓✓	Yes
HmAtELC(K4D)	22.8±6.7	28.3±7.0	S1(A2)-like	✓	Yes
HmAtELC(K3A)	9.4±0.9	15.7±1.1	Intermediate	✓✓	Yes

Notes:

[(a)] Values for kinetic constants are for the ELC hybridised into rabbit skeletal S1 and are shown ± standard error.

[(b)] A tick (✓) indicates that cross-linking was observed. The number of ticks gives a qualitative estimation of the amount of product produced. A cross (×) indicates that no cross-linking could be detected.

[(c)] No rabbit skeletal A2-type ELC was available that was not contaminated with A1-type ELC.

[d] S1(HmAtELC2XP) has an S1(A1)-like K_M and an S1(A2)-like k_{cat}.

Protein engineering is a powerful tool for the investigation of protein function. This power has been brought to bear on the acto-myosin system. Together with kinetic analysis, important questions about the rôle of the ELC in muscle contraction have been answered. Of course, many problems still remain unsolved and new questions are suggested by this work. Access to a source of recombinant protein, along with techniques for analysing effects on the kinetics on the myosin motor will allow further elucidation of the rôle of the essential light chain in fine tuning of the myosin motor. However, a full understanding of its rôle will require an integrated approach involving not only solution kinetics but also *in vitro* motility assays and skinned fibre work.

Appendix

APPENDIX A

A.1 GENE AND PROTEIN SEQUENCES OF THE WILD TYPE HUMAN ATRIAL ESSENTIAL LIGHT CHAIN.

```
          .         .         .         .
   1  ATGGCTCCCAAG AAGCCTGAGCCT AAGAAGGAGGCA GCCAAGCCAGCT
48
      ---------+-- -------+---- -----+------ ---+--------
      TACCGAGGGTTC TTCGGACTCGGA TTCTTCCTCCGT CGGTTCGGTCGA
   1  MetAlaProLys LysProGluPro LysLysGluAla AlaLysProAla
16


    .         .         .         .         .
  49  CCAGCTCCAGCT CCAGCCCCTGCA CCAGCCCCTGCC CCAGCTCCTGAG
96
      -+---------+ ---------+-- -------+---- -----+------
      GGTCGAGGTCGA GGTCGGGGACGT GGTCGGGGACGG GGTCGAGGACTC
  17  ProAlaProAla ProAlaProAla ProAlaProAla ProAlaProGlu
32


     .         .         .         .         .
  97  GCTCCCAAGGAA CCTGCCTTTGAC CCCAAGAGTGTA AAGATAGACTTC 144
      ---+-------- -+---------+ ---------+-- -------+----
      CGAGGGTTCCTT GGACGGAAACTG GGGTTCTCACAT TTCTATCTGAAG
  33  AlaProLysGlu ProAlaPheAsp ProLysSerVal LysIleAspPhe
48


       .         .         .         .         .
 145  ACTGCCGACCAG ATTGAAGAGTTC AAAGAGGCCTTT TCATTGTTTGAC 192
      -----+------ ---+-------- -+---------+ ---------+--
      TGACGGCTGGTC TAACTTCTCAAG TTTCTCCGGAAA AGTAACAAACTG
  49  ThrAlaAspGln IleGluGluPhe LysGluAlaPhe SerLeuPheAsp
64


         .         .         .         .         .
 193  CGGACCCCGACT GGAGAGATGAAG ATCACCTACGGC CAGTGCGGGGAT 240
      -------+---- -----+------ ---+-------- -+---------+
      GCCTGGGGCTGA CCTCTCTACTTC TAGTGGATGCCG GTCACGCCCCTA
  65  ArgThrProThr GlyGluMetLys IleThrTyrGly GlnCysGlyAsp
80


              .         .         .         .
```

```
    241  GTACTGCGGGCC CTGGGCCAGAAC CCTACCAATGCC GAGGTGCTGCGT 288
         ----------+-- -------+---- -----+------ ---+--------
         CATGACGCCCGG GACCCGGTCTTG GGATGGTTACGG CTCCACGACGCA
     81  ValLeuArgAla LeuGlyGlnAsn ProThrAsnAla GluValLeuArg
96
```

. . . . .

```
289  GTGCTGGGCAAG CCCAAGCCTGAA GAGATGAATGTC AAGATGCTGGAC 336
     -+----------+ ----------+-- -------+---- -----+------
     CACGACCCGTTC GGGTTCGGACTT CTCTACTTACAG TTCTACGACCTG
 97  ValLeuGlyLys ProLysProGlu GluMetAsnVal LysMetLeuAsp 112

       .          .          .          .          .
337  TTTGAGACGTTC TTGCCCATCCTG CAGCACATTTCC CGCAACAAGGAG 384
     ---+-------- -+---------+ ---------+-- -------+----
     AAACTCTGCAAG AACGGGTAGGAC GTCGTGTAAAGG GCGTTGTTCCTC
113  PheGluThrPhe LeuProIleLeu GlnHisIleSer ArgAsnLysGlu 128

         .          .          .          .          .
385  CAGGGCACCTAT GAGGACTTCGTG GAGGGCCTGCGT GTCTTTGACAAG 432
     -----+------ ---+-------- -+---------+ ---------+--
     GTCCCGTGGATA CTCCTGAAGCAC CTCCCGGACGCA CAGAAACTGTTC
129  GlnGlyThrTyr GluAspPheVal GluGlyLeuArg ValPheAspLys 144

           .          .          .          .          .
433  GAGAGCAATGGC ACGGTCATGGGT GCTGAGCTTCGG CACGTCCTTGCC 480
     -------+---- -----+------ ---+-------- -+---------+
     CTCTCGTTACCG TGCCAGTACCCA CGACTCGAAGCC GTGCAGGAACGG
145  GluSerAsnGly ThrValMetGly AlaGluLeuArg HisValLeuAla 160

             .          .          .          .
481  ACCCTGGGAGAG AAGATGACTGAG GCTGAAGTGGAG CAGCTGTTAGCT 528
     ---------+-- -------+---- -----+------ ---+--------
     TGGGACCCTCTC TTCTACTGACTC CGACTTCACCTC GTCGACAATCGA
161  ThrLeuGlyGlu LysMetThrGlu AlaGluValGlu GlnLeuLeuAla 176

       .          .          .          .          .
529  GGGCAAGAGGAT GCCAATGGCTGC ATCAATTATGAA GCCTTTGTCAAG 576
     -+---------+ ---------+-- -------+---- -----+------
     CCCGTTCTCCTA CGGTTACCGACG TAGTTAATACTT CGGAAACAGTTC
177  GlyGlnGluAsp AlaAsnGlyCys IleAsnTyrGlu AlaPheValLys 192

       .          .
577  CACATCATGTCA GGGtga  594
     ---+-------- -+----
     GTGTAGTACAGT CCCact
193  HisIleMetSer GlyEnd  198
```

Sequences from Arnold et al., 1988.

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