UNIVERSITY OF BRISTOL

We hereby certify that

WOLFGANG HEINRICH GOLDMANN

having duly satisfied the Examiners appointed by Senate
and having fulfilled all the conditions prescribed
by ordinance and regulations
by resolution of the Council
was awarded the Degree of

DOCTOR OF PHILOSOPHY

on the

18th October 1990

Vice-Chancellor

Registrar

My copy

PRESSURE AND TEMPERATURE PERTURBATION STUDIES OF THE INTERACTION BETWEEN ACTIN AND MYOSIN AND BETWEEN CALCIUM AND TROPONIN C

A dissertation submitted to the University of Bristol in candidature for the degree of Doctor of Philosophy

by

Wolfgang Heinrich GOLDMANN

Department of Biochemistry

University of Bristol

July, 1990

SUMMARY

Coates et al. (1985) have shown that the association reaction of actin with myosin subfragment 1 (S1) consists of at least two steps following the formation of a collision complex;

$$A + M \overset{K_o}{\longleftrightarrow} AM \overset{K_1}{\longleftrightarrow} A\text{-}M \overset{K_2}{\longleftrightarrow} AM$$

The influence of monovalent anions, ionic strength, organic solvents, nucleotide and temperature on this reaction is examined by various kinetic techniques (stopped flow, pressure jump and slow temperature jump).

Increases in ionic strength above 0.1M, the presence of ethylene glycol or dimethylsulphoxide or the addition of nucleotide reduce K_2. In contrast, K_oK_1 is relatively unaffected by these treatments. However, specific monovalent anions above 0.1M do reduce K_oK_1 by increasing k_{-1}. The differential effect of these parameters to stabilize actomyosin intermediates for structural analysis is discussed.

Geeves & Ranatunga (1990) have demonstrated that high pressure augments twitch tension in intact muscle fibres. Studies of troponin C in solution by pressure jump method show that the binding to calcium is not affected by 100atm pressure. This suggests, assuming that the effect of pressure is no different to that in fibres, that muscle is "switched on" to a larger extent per stimulus at high pressure by either increased release of

calcium from the sarcoplasmic reticulum or by a change in the inhibitory function of the regulatory proteins.

The rate binding properties of fluorescent labelled TnC to calcium are investigated by pressure jump and fast temperature jump methods.

PREFACE

The studies presented here were carried out in the Department of Biochemistry, University of Bristol, between February 1987 and July 1990. The work forms no part of any thesis previously submitted and apart from exceptions noted, was carried out unaided.

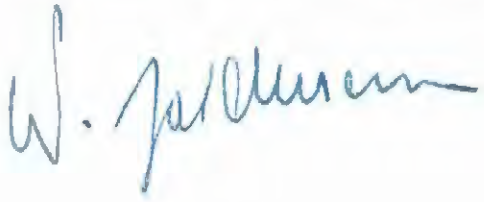
ACKNOWLEDGEMENTS

I gratefully acknowledge the discussion and advice of my supervisor, Dr. M.A. Geeves during the preparation of this thesis and would like to thank him very much for all his help during my research.

I would also like to thank Prof. H. Gutfreund for advice and financial help to carry out this project.

I like to thank Mr. Rick Border and Dr. T.E. Jeffries for technical advice with protein preparations during the early stages of this work, Mrs. M. Bray for clean glassware and Drs. M.A. Geeves, D.J. Halsall and Mr. D. McKillop for the use of the computer software to analyse data. A special thanks goes to Dr. M.A. Geeves, Prof. H. Gutfreund and Drs. N.S. Fortune and J. Hancock for their critical reading of this manuscript.

Finally, I thank Dr. E. Davies, Mr. Paul Connibear and all the others in the Inner Court for the friendly working atmosphere.



PUBLICATIONS

1. The influence of anions, ionic strength and organic solvents on the interaction between actin and myosin subfragment 1. M.A. Geeves & W.H. Goldmann (1990), Biochem. Soc. Transactions, Vol.18, 585-586

2. A "slow" temperature jump apparatus built from a stopped flow machine. W.H. Goldmann & M.A. Geeves (1990). Submitted to Anal. Biochem.

CONTENTS

page

CHAPTER 1 INTRODUCTION

Inherent movement is the prime sign in life. For this and many other reasons, man has shown a perpetual curiosity about the organs of locomotion in his own body and in those of other creatures since the times of Greek anatomists. Indeed, some of the earliest scientific experiments known, concerned muscle and its functions. Although a large number of different techniques have been employed, the problem of energy transduction from chemical reaction into mechanical work is still not fully understood today.

Muscle is a highly efficient and versatile energy-converting device, with remarkable engineering and performance characteristics. The conversion of the chemical energy of ATP into the mechanical energy required to bring about contraction, translocation and propulsion, is still one of the most challenging problems in modern biochemistry.

The study of muscular contraction has encompassed many different scientific disciplines. Biochemists, physiologists, biophysicists, electron-microscopists have all been involved in the investigation of muscle contraction. Most of the recent advances in muscle research have derived from correlation of these disciplines, with the main approaches being structural, mechanical and biochemical.

The work presented in this thesis is concerned with the biochemical events of protein-protein interaction in muscle. Current theories in this field and the contribution to other disciplines will be discussed in this introduction.

MUSCLES

Muscle types

Movement is one of the essential properties of a living organism and is made by cellular contractility. In a unicellular organism, movement is one of several functions of the single cell, but in coelenterates it becomes a specialised function of certain surface epithelial cells. When examining more complex animals, specialised muscle cells can be found, which contract to produce coordinated movements under the control of some type of nervous organization. In vertebrates muscle, cells are further specialised and three distinctive types of muscle may be recognised: smooth muscle, which is regulated involuntarily and shows little well-defined structure; cardiac muscle, which is regulated spontaneously and functions continuously throughout life and skeletal muscle, which is under voluntary control and forms a major part of the body mass.

The morphology and function of skeletal muscle is much better understood than our corresponding knowledge of smooth and of cardiac muscle. While the known differences between these three fundamental muscle types can not be ignored, much of the information on the anatomical structure, on the physiology of contraction and on the biochemistry of action of muscle proves relevant to all three forms of muscle.

This thesis is concerned solely with rabbit skeletal muscle, although biochemical studies have been performed on other skeletal muscles from frogs, chickens, fish and insects.

Muscle structure

The substructure of striated muscle, explored by light microscope, is shown schematically in fig. 1.1. Muscle fibres are composed of longitudinal fibrils, approx 1-2um in diameter, with alternating dark and light bands. The dense bands are birefringent and are called the A (anisotropic) bands; the light bands are known as I (isotropic) bands. Within the I-band lies the dense Z line, about 80nm thick, which is continuous across the width of the fibre, holding the fibrils together and keeping the A and I bands of the many fibrils in register. A package of myofibrils from Z line to Z line is a sarcomere.

COMPONENTS OF MUSCLE

The thick filament

Myosin is one of the principal protein components of the contractile system and comprises of approx 50% of the total protein in skeletal muscles. It is a relatively large (approx 500kDa Mwt.), asymmetric, hexameric protein containing several structural and functional domains (Cooke, 1986). Under denaturing conditions myosin dissociates into two heavy chains of approx 230kDa Mwt. and four light chains of 16-20kDa Mwt. (Harrington & Rodgers, 1984). A diagrammatic representation of the myosin structure is shown in fig. 1.2a.

At physiological salt concentrations myosin aggregates to form bipolar thick filaments, which are insoluble below 0.3M ionic strength (Margossian & Lowey, 1982). The molecule contains

three main regions, which are sensitive to proteolysis. Chymotryptic digestion in the presence of divalent metal ions results in the cleavage of the tail region or "light meromyosin" (LMM) and "heavy meromyosin" (HMM). LMM is 86nm long and insoluble at physiological ionic strength while the soluble HMM consists of the "S2" region, which is 60nm long and the "S1" head region (Stewart & Edwards, 1984). The junction between the "S2" region and the globular "S1" head region is susceptible to proteolysis in the absence of divalent metal ions.

LMM consists of two heavy chains in a coiled-coil α-helical structure, which is highly repetitive (Karn et al., 1983). The hydrophobic residues on the inside of the helix form the interface between the two strands of the coiled-coil. The amino acid sequence of this portion has provided at least a partial explanation of the forces that hold the core of the thick filament together (McLachlan, 1984).

The "S2" section is thought to contain the flexible region, which links the "S1" with the rigid tail region. The sequence of the rod basically favours the strong α-helical secondary structure, however, weaker parts have been found at the junction between LMM and "S2". This region is susceptible to proteolysis and thought to be essential for the functioning of the protein, allowing the head group to interact cyclically with the actin filament (Applegate & Reisler, 1984).

The "S1" head region can be cleaved into three segments with molecular weights of 50, 25 and 20kDa, respectively (Balint et al., 1978). The N-terminal 25kDa fragment binds nucleotide and

has sequence homology with other nucleotide binding proteins. The 50kDa and 20kDa segments can be cross-linked to actin (Mornet et al., 1981a;b; Sutoh, 1983; Vibert & Cohen, 1988). The 20kDa region extends to the "swivel", an area, which connects the head with the rod and which binds both of the light chains. The region contains two highly reactive sulphydryls, SH-1 and SH-2 (fig. 1.2b).

Each "S1" head contains two classes of light chains; the P-light chain, which can be phosphorylated and the alkali A-light chain, which exists in two different forms referred to as A1 and A2. The P-light chain is involved in the regulation of contraction (Adelstein & Eisenberg, 1980). In smooth and some non-muscle cells this is achieved via phosphorylation of this chain, and in skeletal and cardiac muscle, the contraction is modulated by the phosphorylation. The A-light chain was first thought to be essential for the myosin ATPase activity, however, now it is known, myosin devoid of both light chains retains an actin activated ATPase activity similar to intact myosin. The two isoforms of A-light chains, A1 and A2 have been found in skeletal muscles, which confer different ATPase activities (Sivaramakrishnam & Burke, 1982; Wagner & Stone, 1983) and actin binding on S1 at low ionic strength (Trayer et al., 1985).

Winkelmann et al. (1985) have crystalized S1 and determined the overall length of the molecule to be 16nm. Electron micrographs of the crystals have indicated two domains, one of higher and one of lower density. The higher density region has a prominent mass at one end with dimensions of 6 x 4nm. The region of lower density forms a curved neck region and extents from one

end of the mass. This mass probably comprises of 20kDa portion of the heavy chain and the two light chains (Baker & Winkelmann, 1986) (fig. 1.2c).

OTHER COMPONENTS OF THE THICK FILAMENT

Although myosin is the main component of the thick filament of vertebrate striated muscle, it is not the only one. In electron micrographs of A bands a set of 11 stripes, 43nm apart, may be seen in each half of the A band, occupying a region 0.25-0.50μm from the centre (Craig, 1977). Some, but not all, of the components have now been identified: B-protein, which is a component of the M-line; C-protein; F-protein has been identified as the enzyme phosphofructokinase; H-protein and X-protein (Starr & Offer, 1982). A group of giant myofibrillar proteins are present in abundance in a wide range of vertebrate and invertebrate striated muscles: titin and nebulin. These proteins are unusual not only in their giant sizes, but also in their solubility and their localisation in the sarcomere. The function of all these proteins is not fully understood today, but they could be involved as regulators of cross-bridge movements, in a mechanical or structural function or in filament assembly (Trinick et al., 1984; Wang & Greaser, 1985; Marayama et al., 1989).

THE THIN FILAMENT

Actin is a globular protein with a single polypeptide chain, which in skeletal muscle is composed of 375 amino acids and has a

molecular weight of 42 kDa (Collins & Elzinga, 1975). It forms the backbone of the thin filament and has been shown to occur in every eukaryotic cell studied sofar (Clarke & Spudich, 1977). The complete amino acid sequence has been determined and showed a remarkable degree of homology in muscle and non-muscle cells with only a few amino acid substitutions predominantly near the N-terminus of the chain (Vandekerckhove & Weber, 1979).

At physiological salt concentration, actin polymerizes to filamentous (F)-actin. F-actin is a double-stranded helix linked by non-covalent bonds and with 13-15 molecules of globular (G)-actin for one full turn (fig. 1.3a). In solution the filaments are in dynamic equilibrium with the monomeric actin and the association of the monomers occur preferentially from one end of the filament.

In (1981) Suck et al. determined the structure of monomeric actin from rabbit skeletal muscle by X-ray crystallography at 6 Angstrom resolution. The structure showed that the molecule is asymmetric with dimensions of approx 7nm x 4nm x 3nm and composed of two different size domains, which are separated by a cleft. Recent reconstructions of actin polymers have resolved the actin monomer into a major and a minor domain, similar to those determined from X-ray crystallography (O'Brien et al., 1983) (fig. 1.3b), despite the ambiguity about the situation of the domains nearest to the filament axis (Egelman, 1985). Attempts to determine an actin structure at 3 Angstrom resolution have been made by Kabsch & Holmes (1990) and preliminary results have been published.

dimer = 11 subunits comprised of molecules each of which comprises two molecules of A

OTHER COMPONENTS OF THE THIN FILAMENT

Tropomyosin

Tropomyosin (Tm) is a key protein in the regulation of muscle contraction and is found in a wide range of non-muscle cells. It exists as a dimer of two polypeptide subunits, which associate in register in a coiled-coil fashion (Graceffa & Lehrer, 1980). The subunits of 284 amino acid residues and 33kDa each have very similar amino acid sequences (Hodges et al., 1972). Crystals of tropomyosin have been formed to about 4 Angstrom resolution, in which the 400 Angstrom long molecule appears to bonded head-to-tail to form continuous filaments. X-ray diffraction pattern showed second order helical layer lines arising from the 140 Angstrom pitch of the two-stranded coiled-coil, which suggests that Tm can bind actin by winding around the actin filament. The eight or nine residue head-to-tail overlap of tropomyosin molecules is crucial to Tm polymerization and binding to actin (Phillips et al., 1986) (fig. 1.4a).

Troponin

Ebashi & Kodama (1966) and Endo & Ebashi (1966) first demonstrated that the Ca^{2+} dependence of actomyosin ATPase activity required a protein, which they called troponin (Tn). The exact composition of troponin was uncertain for several years until Greaser & Gergely (1971; 1973) established that troponin consists of three non-identical subunits, which led to the following terminology: (TnT), which binds tropomyosin and attaches the troponin complex to the thin filament, (TnI), which

inhibits the actin.subfragment 1 (acto.S1) ATPase and (TnC), which binds Ca^{2+}. The subunits interact with tropomyosin and are situated one third of the distance from the carboxyl-terminal end of the (Tm) molecule (fig. 1.4b).

Troponin T

Troponin T (TnT) is the largest subunit of troponin with a calculated molecular weight of 30,503 Daltons and 259 amino acid residues (Pearlstone et al., 1976). This globular protein with a rod-shaped tail is highly polar near the amino and carboxyl termini and links the troponin complex to tropomyosin. It has direct interactions with TnI, TnC and tropomyosin and confers a Ca^{2+}-sensitive inhibition of ATPase activity (Greaser & Gergely, 1971). Crystals of TnT and Tm have been formed to 17 Angstrom resolution by White et al. (1987).

Troponin I

Troponin I (TnI) has a calculated molecular weight of 20,864 Daltons and consists of 179 amino acids (Wilkinson & Grand, 1975). This rod-shaped molecule has two segments, which are significant in the regulatory thin filament interactions. Functionally, TnI inhibits the actomyosin Mg^{2+}-ATPase. In the absence of tropomyosin or troponin, one TnI molecule per actin monomer is required to inhibit activity. This inhibition is abolished by the presence of TnC, irrespective of $[Ca^{2+}]$ (Greaser & Gergely, 1971).

Troponin C

Troponin C (TnC) is composed of 159 amino acid residues with

a calculated molecular weight of 17,846 Daltons (Collins et al., 1973). Crystal structures to a resolution of 2.2 Angstroms have been produced, which have revealed two distinct domains: one containing the amino-terminal Ca^{2+}-specific sites and the other containing the carboxyl-terminal Ca^{2+}-Mg^{2+}-sites. These sites are separated by a long nine turn α-helix, producing a dumb-bell shaped molecule about 75 Angstrom long (Herzberg & James, 1985) (fig. 1.4c). Potter & Gergely (1975) had found four Ca^{2+}-binding sites on (TnC), which fitted best two classes of sites: two low-affinity sites that selectively bind Ca^{2+} over Mg^{2+} (Ca^{2+}-specific sites; K_{Ca} approx 10^5 M^{-1}) and two high-affinity sites that bind Ca^{2+} and Mg^{2+} competively (Ca^{2+} - Mg^{2+} sites; K_{Ca} approx 10^7 M^{-1}; K_{Mg} approx 10^3 M^{-1}). The interactions between TnC and TnI and actin are central to the Ca^{2+}-dependent regulation of contraction. TnC and TnI interact both in the presence and absence of Ca^{2+}.

MUSCLE CONTRACTION

Cross-bridge theories

A vertebrate skeletal muscle fibre is made up of many sarcomeres, which are approx 2.4um long and contain an interdigitating array of thick and thin filaments stacked end-to-end. On muscle shortening, the thick and thin filaments slide passed each other, leaving the length of filaments constant. This was first observed by H.E. Huxley & Hanson (1954), using interference microscopy and by A.F. Huxley & Niedergerke (1954), using phase contrast microscopy and led to the sliding filament

model of muscle contraction.

In (1957) A.F. Huxley proposed the existence of an elastic element attached to one filament, which could cyclically interact with the other filament, depending on the tension in the fibre and the presence and absence of ATP. H.E. Huxley (1957) identified these elastic elements by high-resolution electron micrographs as "cross-bridges" between neighbouring thick and thin filaments, which were later recognized as the S1 heads of myosin (fig. 1.5a).

In the (1957) theory of A.F. Huxley, force production was accommodated by making the rates of attachment and detachment of force generating links (now cross-bridges) sensitive to position. The rate constants for these two processes were believed to be dependent on the relative axial positions of the myosin-based projections and the actin-based attachment sites. Attachment would be faster than detachment in regions where cross-bridges generated positive tension, but detachment would be much faster from positions, in which the cross-bridges exerted a force opposed to the normal shortening motion. Cross-bridges could not attach directly in positions that exerted negative tension and could reach such positions only through the sliding of the filaments relative to each other during shortening. With the values for attachment and detachment, it was possible to fit quantitatively the experimental data to the mechanical and energetic steady-state properties of muscle that were available at the time. However, rapid transient changes seen in muscle were not included in this theory.

Huxley & Simmons (1971) added this extra feature to the

cross-bridge representation used in the A.F. Huxley (1957) theory. The attached cross-bridge head (S1) can now take up a number of different conformations. Rapid tension transients are explained by an elastic element in the cross-bridge arm prior to an orientation change of the cross-bridge (fig. 1.5b).

A widely accepted theory, today, about the mode of action of cross-bridge contracting muscle can be summarized as follows:

a) Cross-bridges cyclically attach to and detach from thin filaments.
b) Force can be generated in the attached cross-bridge, pulling the thin filaments along in the direction that produces muscle shortening.
c) ATP hydrolysis is coupled to such a cycle, providing the energy of contraction.

This theory has been developed to describe quantitatively many mechanical, energetic, biochemical and structural properties of muscle. It is convenient to discuss experimental results in these terms (see also Woledge et al., 1985).

BIOCHEMICAL STUDIES

Many studies have been carried out on muscle proteins with biochemical techniques and correlated with structural and mechanical properties in fibres (Hibberd & Trentham 1986; Irving, 1987). Proteolytic digestion of myosin has made the biochemical approach a powerful tool. By using these methods, myosin retains

its actin binding and ATPase activity and has proved valuable in attempting to elucidate the mechanism of muscle contraction. In interpretating biochemical results, it is important to take into account, the effects of solubilising and digesting muscle proteins.

A common criticism of biochemical studies of actin and myosin subfragment 1 (S1) is that the local concentration of proteins will be much higher in the filament. Further, that solution studies are normally performed at ionic strengths lower than physiological conditions. Also steric contraints in the filament will affect the rates of some of the muscle protein interactions. As proteins in solution can do no work, free energy of ATP hydrolysis must be dissipated as heat. Therefore, solution studies are often compared to muscles contracting under no load. Experiments to compare the solution studies with <u>in vivo</u> observations will be discussed (Geeves, 1990; Millar & Homsher, 1990).

THE LYMN-TAYLOR SCHEME

The basic model for the biochemical mechanism of the actomyosin ATPase reaction is the Lymn-Taylor scheme (Lymn & Taylor, 1971). This scheme gives a relatively simple view of how the chemistry of ATP hydrolysis could be related to cycling cross-bridges. The basis of the scheme is as follows: Following ATP binding to actomyosin (AM), actin (A) dissociates from the (AM.ATP) complex faster than ATP is hydrolysed, so the intermediate (M.ATP) is formed. After ATP hydrolysis the myosin products complex (M.ADP.Pi) can rebind to actin, so that there is

a rapid equilibrium between the states (M.ADP.Pi) and (AM.ADP.Pi). Release of the products occurs much faster from (AM.ADP.Pi) than from (M.ADP.Pi), giving the strongly bound (AM.ADP) or (AM) states and completing the cycle and giving it a direction. Fig. 1.5c shows the structural correlations that were proposed to accompany the biochemical events.

THE MODEL OF EISENBERG & GREENE

Stein et al. (1979) tested the Lymn-Taylor scheme and showed that it does not describe the pathway of ATP hydrolysis in solution accurately under all conditions. It was observed that, when actin concentration is increased to nearer the concentration in muscle, it could reversibly bind to all intermediates of the ATPase pathway. Furthermore, ATP binding to actomyosin at low ionic strength does not necessarily lead to actin dissociation before ATP hydrolysis. In contrast to the Lymn-Taylor scheme, the reaction (AM.ATP $\longleftrightarrow$ AM.ADP.Pi) now became an important step in the mechanism. In both these states, myosin is weakly bound to actin; each is in rapid equilibrium with the corresponding myosin state (M.ATP or M.ADP.Pi).

Eisenberg & Greene (1980) proposed a cross-bridge model on the basis of the kinetic scheme by Stein et al. (1979) (fig. 1.5d). Free cross-bridges are in rapid equilibrium with actin-bound cross-bridges. In the weak-binding 90° conformation of the cross-bridge two kinetic events occur (ATP hydrolysis and a "rate-limiting" step). This is followed in the cross-bridge cycle by the transition from the weak-binding 90° to the strong-binding

45° conformation with tension development, Pi release and force production, which is different in muscle and solution as there is no restraint in solution. It was suggested by Eisenberg & Greene (1980) that in muscle tension development and movement occur in two steps. Both steps are a result of the nature of attachment of many cross-bridges instead of one. During tension development a strained 45° attachment exists, exerting positive tension, which is only relieved by the gradual and relative movement of the filaments. The release of ADP from the cross-bridge is slow only until the cross-bridge reaches the stable 45° conformation, then ADP release becomes as rapid as in solution. With the release of ADP, ATP binds to the cross-bridge causing it to return to the 90° state. This state exerts negative tension, but M.ATP detaches rapidly from AM.ATP and no significant tension is developed.

THE MODEL OF GEEVES, GOODY AND GUTFREUND

The transition between weak and strong actin binding states were discussed further by Geeves et al. (1984) and interpreted in the following two step model;

$$A + MN \underset{}{\overset{K_1}{\rightleftharpoons}} A\text{-}MN \overset{K_2}{\rightleftharpoons} AMN$$

(A=actin; M=myosin; N=nucleotide)

In the first step actin binds to form a weakly "attached" ternary complex with an association constant of 10^3 to 10^4 M^{-1} and then isomerizes to a "rigor-like" complex. It was proposed

that the first step is relatively independent of nucleotide. The second step, however, was dependent upon the nucleotide present at the myosin subfragment 1 (S1) nucleotide binding site. The equilibrium constant (K_2) of the second step dictates whether the ternary complex exists in either the strong or weak binding state. If $K_2 > 1$ the strong complex predominates, if $K_2 < 1$ the weak complex predominates. The isomerization step is believed to be related to the force generating event in muscle. Eisenberg & Greene (1980) had suggested the transition from the weak 90^{o} state to the strong 45^{o} state to be responsible for tension generation.

Geeves et al. (1984) discussed the kinetic data available at the time in terms of two different models: a direct coupling model suggesting that the steps in the ATPase cycle are directly responsible for conformational changes that represent tension generation in muscle; and an indirect coupling model, in which changes in affinity of actin for myosin at different stages throughout the ATPase cycle determine the time, the myosin molecule would spend in the attached and rigor-like state. The change in both environments is followed by the power stroke.

Several pieces of indirect biochemical evidence for the existence of two states of actin and myosin subfragment 1 (S1) have been provided by various workers with different techniques and under different conditions.

In (1980) Sleep & Hutton studied the oxygen exchange of inorganic phosphate (Pi) and ATP in the presence of acto.S1 and demonstrated that the exchange was greater with hydrolysis of ATP

than when ADP and Pi were only present in the system. The existence of two ADP states were proposed under steady state conditions, one of which could not be formed upon addition of ADP. The kinetics of the interaction between actin and S1.ADP or S1.ethenoADP has also been studied by stopped flow (Marston, 1982; Trybus & Taylor, 1980; Siemankowski & White, 1984; Rosenfeld & Taylor, 1984) and by pressure relaxation methods (Geeves & Gutfreund, 1982). These studies were consistent with a simple one-step binding reaction, preceded by a rapid pre-equilibrium or collision complex.

In the pressure jump study by Geeves & Gutfreund (1982), the interaction of acto.S1 binding was monitored by following the changes in light-scattering. The sensitivity of this method, however, limited the study to conditions (at high salt concentration or in the presence of ADP) where the association constant was between 10^4 and 10^6 M^{-1}.

The use of actin labelled at Cys 374 with a pyrene fluorophore, which has no significant effect on the binding, has enabled characterization of additional acto.S1 and acto.S1-nucleotide complexes (Criddle et al., 1985; Geeves et al., 1986). Employing the specific pyrene probe increased the sensitivity of the pressure jump studies, allowing relaxations at physiological ionic strength and in the absence of nucleotide to be observed. Coates et al. (1985) provided the first direct evidence for this model. It was revealed that two relaxations of acto.S1 were apparent corresponding to two steps in the actin binding reaction with only significant pyrene fluorescence quench on the second, pressure-sensitive step.

More recent work demonstrated that the same two states can be identified when nucleotide or nucleotide analogues are bound to myosin and that the equilibrium constant (K_2) is particular sensitive to the nature of the occupancy of the nucleotide site on myosin (Geeves et al., 1986; Geeves & Jeffries, 1988; Geeves, 1989). The model proposed by Geeves et al. (1984) is shown in fig. 1.6 with experimentally determined or estimated values for (K_1) and (K_2).

RELATION OF BIOCHEMISTRY AND STRUCTURAL STUDIES

The biochemical evidence of intermediates in the actomyosin ATPase cycle might be testable by structural methods, e.g. the fraction of cross-bridges that are attached to actin in a particular mechanical or biochemical state or the existence of two or more configurations of the attached cross-bridge.

Some of the most detailed information about cross-bridge structure has come from electron microscopy. X-ray diffraction is used to gain further information about periodic structural features, whilst X-ray scattering has been used to investigate aperiodic charateristics such as cross-bridge orientation (Irving, 1987). The reactive sulphydryl (SH-1) of myosin subfragment 1 (S1) sites on the cross-bridges can be modified by introducing extrinsic probe molecules, which can be used for optical studies of structure changes. In addition to fluorescence and dichroism studies, extrinsic probes have been used for electron spin resonance (ESR) and electron paramagnetic resonance (EPR) studies of muscle fibres (for references see Cooke, 1986).

Results from electron microscopy in the absence of ATP of actin filaments with myosin (S1) bound to them indicate that heads make an angle of about 45° with the filament axis. X-ray scattering confirms this orientation of 45° to the filament axis (Poulsen & Lowy, 1983) and X-ray diffraction suggests stereospecific attachment of cross-bridge heads. Results from experiments using spin or optical probes attached to S1 support this view. The presence of ADP in rigor muscle has no effect on the mechanical properties in X-ray pattern or orientation of a spin label on myosin (SH-1). Local changes in myosin conformation on addition of ADP have been detected by probes attached to SH-1 (Burghardt et al., 1983).

Actin-based layer lines in electron micrographs of relaxed muscle are weaker than in rigor muscle, suggesting cross-bridge detachment from actin. Extrinsic probes detect a greater disorder of orientation in relaxed than in rigor muscle. X-ray and birefringence measurements estimate an average cross-bridge head angle of 30° with the filament (Irving & Peckham, 1986).

In contracting muscle, X-ray diffraction result suggest 60-80% of cross-bridge attachment to actin (Huxley & Kress, 1985). EPR spectra of isometric contracting muscle indicate 20% of the spin probes on myosin SH-1 are in rigor conformation and 80% remain disordered and mobile. Fluorescence ATP analogue measurements showed 40% of cross-bridge heads in rigor conformation. Differences in these results are interpreted as possible two types of attached cross-bridges.

Clearly, the available structure data are inadequate. It may be necessary to develop new methods to probe the orientations,

mobility or interactions of several defined sites on the cross-bridge to answer questions of structural basis of tension generation (Cooke, 1986).

THE RELATION OF BIOCHEMISTRY TO MECHANICS

An experimental system that allows mechnical observations, while the biochemical milieu of the myofilament is altered, is that of "skinned fibre" preparations: The surface membrane of the muscle fibre is either chemically or mechanically removed and placed in solutions that mimic the intracellular environment. The de-membranated muscle cells with largely intact myofilaments has provided new information about the relationship between mechanical performance (tension development, shortening velocity and response to rapid perturbations) and the chemical environment of the myofilaments. However, caution is required in extrapolating from skinned fibres to intact muscle (Woledge et al., 1985).

The rates of ATP binding and hydrolysis in fibres were measured by Sleep & Smith (1981) and correlated with data from solution studies. The results are ambiguous as protein concentration in fibres and diffusional delays of ATP concentration could not be controlled. Much of the information about actomyosin in muscle has, therefore, come from studies of steady-state and transient mechanical properties of muscle fibres in solutions of various maintained compositions. Cooke & Pate (1985) observed an increase of isometric tension in the presence of ADP, which was consistent with ADP inhibiting the rate, at

which ATP bound to a tension bearing state. The influence of the products of the ATPase reaction (ADP, Pi) on the cross-bridge cycle in muscle has also been explored, suggesting that ADP release could limit the shortening velocity in muscle (Siemankowski & White, 1984; Siemankowski et al., 1985). Oxygen exchange studies were used to elucidate the stages of myosin ATPase in solution and in fibre (Webb et al., 1986). It was observed that exchange occurs at a greater rate in fibres than in solution of purified proteins. It has become possible to study the kinetics of transitions between biochemical states more directly by imposing a rapid step change of ATP concentration within a muscle fibre. This was achieved by allowing ATP protected by a photolabile group, "caged" ATP, to diffuse into the fibre, then illuminating the fibre with an intense pulse of UV-light to generate ATP rapidly in millimolar concentrations (Goldman et al., 1982; Dantzig et al., 1987). Results from these studies indicate that major steady-state intermediates in isometric muscle must have bound hydrolysis products. Time-resolved X-ray diffraction allows the movement of muscle components to be observed directly (Kress et al., 1986) and results indicate a movement of mass towards the thin filament prior to tension increase. More recently, pressure perturbation studies on whole muscle fibres have been carried out and results support the conclusions from solution studies that increased pressure is perturbing a cross-bridge event (Geeves & Ranatunga, 1987; 1990; Fortune et al., 1988; 1989).

A MODEL OF CROSS-BRIDGE ACTION

Based on the models of A.F.Huxley (1957) and Eisenberg & Greene (1980), Pate & Cooke (1989) incorporated all aspects of steady-state kinetics and physiological data obtained from de-membranated muscle fibres into a new model of energy transduction.

The authors explain their model, using one single pathway with five states for ATP hydrolysis: three actomyosin attached states (A.M.ADP.Pi; A.M.ADP; and A.M) and two detached states (M.ATP; M.ADP.Pi). This model accounts for the relationship between ATP concentration, steady-state ATPase rate and isometric tension in contracting fibre with a reasonable set of assumptions.

A basic assumption of this model is that the kinetic cycle of ATP hydrolysis is the same in fibres and solution. The free energies of the detached cross-bridge states are not dependent on the relative actin-binding sites. The (A.M.ADP.Pi) state is mechanically the same regarding stiffness, but exerts little force, and the Pi release is seen as the driving force for muscle shortening. Most force is produced by the A.M.ADP state and the rate-limiting step in isometric muscle is the ADP release. The model also defines a linear relationship between the log of Pi concentration, isometric force and ATPase rate.

Premises made in this model conform with most of the data and interpretations of the past few years. However, some ideas and data challenge the traditional view on energy transduction; e.g. the tight coupling between cross-bridge attachment-detachment

cycles and ATP hydrolysis; the cooperative effects between the two independent myosin heads on the energetics of energy transduction mechanism (for all references Pate & Cooke, 1989).

ENVIRONMENTAL CHANGES OF A SOLUTION CONTAINING ACTIN AND S1: THE EFFECT ON THE STRUCTURE, ACTIVITY AND BINDING OF PROTEINS

The understanding of the interaction of actin and myosin under various experimental conditions is important in the study of muscle function. A great amount of evidence has accumulated to illustrate that the interaction of ions or solvents with muscle proteins plays an important role in the contractile process and, therefore, deserves special study. It has been shown that the presence of ions or solvents influence the conformation, the ATPase activity and other physiochemical properties of the actomyosin complex (for all references Stafford, 1985).

Over the last 20 years a considerable body of research has been concerned with the effect of various salts on the conformational stability of a variety of biological macromolecules and macromolecular assemblies in general. The effect was viewed as a shifting, by the added salt, of the transition boundary between an ordered macromolecular structure (in which residue-residue contacts are thermodynamically favoured for at least the groups comprising the "interior" of the macromolecule) and a disordered or "random" coil state, in which residue-solvent contacts for essentially all of the constituent groups (Von Hippel & Wong, 1962). The phase transition boundary may be shifted towards either higher or lower temperatures

relative to the transition temperature of the macromolecule in a dilute aqueous buffer system, depending on whether the added ions tend to stabilize (thermodynamically favour) the ordered or the disordered forms of the macromolecule. The effects of the individual ions on the macromolecular stability were reported to be independent and additive and generally follow the classical Hofmeister series (Hofmeister, 1888; Von Hippel & Schleich, 1969a;b).

Major work has been carried out by various workers on how these effects are brought about. Studies on macromolecules have

made it clear, that the effects do not depend strongly on details of macromolecular conformation or chemistry (Nandi & Robinson, 1972a;b). Therefore, the emphasis of research has turned to an examination of the properties of small molecules e.g. collagen (Von Hippel & Wong, 1962), DNA (Ross & Scruggs, 1964) and myosin (Warren et al., 1966).

The two major forces, which alter the state of interaction of aqueous solutions of biological molecules, are hydrophobic and electrostatic. They play an important role in the transfer of reactants from the bulk-water phase to the surface of the enzyme protein. Further, their interaction with specific protein groups and concomitant conformational changes of the protein make a large contribution to the thermodynamic state of the system. As the accompanying thermodynamic changes provide a useful insight into the processes involving biological molecules, it allows comparison with information on mechanical changes obtained by other analytical methods (Kodama, 1985).

With the development of kinetic techniques for the study of biological systems, much attention has been directed towards the elucidation of distinct intermediates, their rates of interconversion and the characterization of physical properties. The wide range of kinetic techniques today, allows the observation of reactions with different lifetimes and intermediates. Stopped flow, temperature jump and pressure relaxation techniques are the most widely used. Relaxation methods have the advantage over flow techniques, as the time taken to change the concentration of the reactant is not limited by mixing time, which is approx 1msec. Flow, mixing and flow

stopping artefacts are also avoided using relaxation techniques. Temperature jump reactions can be initiated within the nano -and microsecond range and permit observations up to 1sec. In contrast, pressure relaxation reactions can only be observed after 25usec but with no upper time limit. Further advantages over temperature perturbation technique are: no limit of solvent composition, reproducible reactions in rapid succession and no protein denaturation under 1000atm.

Volume changes during biochemical reactions in aqueous media are largely due to changes in solvent structure due to the interaction with solute molecules (Gutfreund, 1972). The formation and disappearance of charged groups accessible to water result in considerable volume changes and hence processes involving such exposed ionising groups show a marked pressure-dependence. These effects can be due either to conformation changes, which result in exposure of a charged group or to pressure-induced change of the pK of a group linked to a conformational rearrangement. Studies of the effect of pressure over a range of conditions allow one to distinguish between the different causes of electrostriction, ionisation, breaking of salt bridges and exposure of buried charged groups. Hydrogen bond transfer from an intrinsic to a water bonded state is generally associated with a small negative volume change.

The aim of relaxation techniques is to perturb the equilibrium so that subsequent rapid reactions can be studied. The perturbation can be mediated through an indirect effect such as the rapid change in pH or of the concentration of some other

reactant species. Studies of amplitudes of relaxations under different conditions can elucidate the volume changes and thermodymanic characteristics of the reaction steps. The amplitude of the change in equilibrium for a pressure change is given by;

$$(\partial \ln K/\partial P)_t = -\Delta V^o/RxT$$

For small pressure changes this approximates to;

$$\Delta K/K = -\Delta P \times \Delta V^o / R \times T$$

(K = equilibrium constant; P = pressure; V^o = standard volume change; R = gas constant; T = absolute temperature)

The study of protein assembly from monomeric units is of interest both because it can give information about the structure and the forces involved in their stability. The potential of pressure relaxation for the study of the assembly of myosin polymers has been demonstrated (Davis & Gutfreund, 1976).

Geeves & Gutfreund (1982) reported that pressure relaxation could be used as a tool to study the mechanism of acto.S1 interactions. Results demonstrated the potential of this method for studying the rate of equilibration of the attachment of S1 for actin by light scattering under various conditions. It was shown that the binding reaction in the presence of ADP, which was treated in terms of a single-step reaction, was sensitive to ionic strength, temperature and pH changes. Similar observations were made for the binding of actin to S1 by the stopped flow

method in the absence of nucleotide (White & Taylor, 1976; Konrad & Goody, 1982; Marston 1982). The marked ionic strength effect on the forward rate constant was interpretated as a significant contribution from charged groups in the binding reaction, in which electrostatic interactions play an important role. The large temperature dependence of the binding indicated a conformational change in the protein and data from the Arrhenius plot suggested an entropy controlled reaction. Konrad & Goody (1982) also interpreted the association of actin and myosin as an entropy driven process. Large values for ΔS^{o} are normally expected for reactions with significant volume changes. Substantial volume changes usually reflect changes in the ordering of water due to changes in the number of exposed charged or hydrophobic groups (Eagland, 1975). This evidence indicated a more complex binding reaction, and the high activation energy (E_a approx 107 kJ/mol) suggested that the first order isomerzation is preceded by a faster diffusion controlled reaction. Further evidence for a more complex reaction has come from the analysis of the observed amplitudes.

The use of fluorescent labelled actin allowed the interaction of actin S1 to be monitored at physiological ionic strength and in the absence of nucleotide by pressure relaxation methods (Coates et al., 1985). Two relaxations were identified, which represented two events in the interaction of actin with S1. However, it was suggested that the first step may be more complex as the reaction did not have the properties associated with a diffusion controlled reaction. Therefore, this step was

considered to comprise the formation of a collision complex before the attached state is formed;

$$A + M \overset{K_o}{\longleftrightarrow} (A\ M) \overset{K_1}{\longleftrightarrow} A\text{--}M \overset{K_2}{\longleftrightarrow} AM$$

and with no direct information on the collison complex, the reaction was discussed in terms of two steps ($K_1' = K_o \times K_1$). It was demonstrated that ionic strength, temperature and organic solvent had a marked effect on the two individual binding steps and that the second step was sensitive to hydrostatic pressure and caused quenching of pyrene fluorescence. The size of perturbations suggested that the equilibrium had an associated volume change of approx 100 cm^3 per mol.

Volume changes of this magnitude normally indicate a change in the hydration sphere of the system caused by the exposure of charged groups to the solvent and must be seen as evidence for a substantial change in conformation of the protein complex or a change in the contact area between the two proteins.

Thermodynamic data of the binding of ATP to myosin subfragment (S1) (Biosca et al., 1983) and acto.S1 (Millar & Geeves, 1983) revealed a large increase in entropy at low temperature. The results were interpretated in terms of temperature induced conformational change in the protein. The entropic control on the free energy is normally the consequence of the hydrophobic interaction dominating the association process at low temperature. The enhancement of solvent structure by the dissolution of non-polar groups in aqueous solution is greatest

at low temperature, and the tendency for hydrophobic association is increased because of this (Ross & Subramanian, 1981).

Many laboratories have used organic solvents to elcuidate the structure of the cross-bridge intermediates in the actomyosin ATPase cycle. The use of organic solvent has been known to weaken the binding of the acto.S1 complex (Marston & Tregear, 1984). Tregear et al. (1984) reported a shift in the S1 structure in the presence of 40% ethylene glycol towards the weak binding conformation. Increasing salt concentration and ethylene glycol concentration furthered this process. Recently, (Mushtaq & Greene, 1989) reported a similar event for controlled actin. No significant effect of 40% ethylene glycol was observed upon the interaction of ATP with acto.S1 (Millar & Geeves, 1983). Geeves & Jeffries (1988) observed that the acto.S1 complex in the presence of 40% ethylene glycol and ADP exists to less than 30% in the weak binding conformation.

The aim of this thesis is to examine the effect of different anions, temperature, solvents, ionic strength and nucleotide in order to understand the nature of the acto.S1 reaction in each step. The differential effect of these parameters could be used to trap actomyosin in the weakly attached state preferentially, facillitating structural studies of the two states.

Results from these studies are evaluated in terms of the two step binding model of Geeves et al. (1984).

CHAPTER 2 MATERIALS AND METHODS

REAGENTS

General reagents were purchased from BDH Chemicals Co., Boehringer Mannheim GmbH., Sigma Chemicals Co. and Aldrich Fine Chemicals, unless otherwise stated. DTT was obtained from Park Scientific and Urea (Enzyme Grade) was supplied by Bethesda Research Lab. (BRL). Fluorescence probe (N-(1-pyrene)iodoacetamide was purchased from Molecular Probes Inc. and N-dansylaziridine was obtained from Pierce Chemicals Co. The chromophoric indicator arsenazo III was a gift from Dr. D. Yates, Bristol University.

GENERAL BUFFERS

Experiments were generally performed at pH 7.0. In pressure jump studies imidazole (pK 7) was used because of its pH stability upon pressure perturbation. Titrations were mainly conducted in tris (pK 8) or cacodylate (pK 6.27) buffers, due to occasional difficulties with background fluorescence caused by impurities in imidazole preparations. Slow and fast temperature jump experiments were usually carried out using cacodylate since this buffer is reasonably insensitive to changes in temperature.

All buffers contained 1mM sodium azide to prevent bacterial and fungal contamination. Double distilled water was used for all buffers. The glassware was rinsed with double distilled water after washing. Magnesium ions were used as described in the appropriate sections.

CHAPTER 3 THE EFFECT OF IONIC STRENGTH, SOLVENTS, TEMPERATURE AND MONOVALENT ANIONS ON THE INTERACTION OF ACTIN TO MYOSIN SUBFRAGMENT 1 (S1)

INTRODUCTION

Coates et al. (1985) have shown, using pressure relaxation method that the association between myosin subfragment 1 (S1) and actin occurs in a two-step binding preceded by the formation of a collision complex. In the first step S1 binds to actin to form a weakly "attached" state and then isomerizes to a "rigor-like" complex. This transition between the two states is believed to be closely associated with force generation in muscle (Geeves et al., 1984). In their study, Coates et al. (1985) also showed that temperature, ionic strength and organic solvent had a significant influence on the individual steps of the reaction.

It is possible that S1 binds to actin at two distinct sites and that S1 can bind at one site to give an "attached" state and binding at both sites is required to give the "rigor-like" state. Previously, Mornet et al. (1981a) had investigated the topography of an acto.S1 rigor complex by cross-linking technique and results showed that the myosin head S1 enters into a "van der Waal" contact with two neighbouring actin monomers. Structural evidence that myosin heads may interact with two sites on F-actin was produced by Amos et al. (1982). The apparent existence of two independent sites of contact between S1 and F-actin in the rigor state suggested to these authors that the interaction during an active stroke might involve different S1 binding configurations.

(cross-linking = joining of polymer molecules to each other by valence bonds. - v. d. Waal attraction)

CHAPTER 4 THE EFFECT OF DIFFERENT ANIONS AND IONIC STRENGTH ON THE ISOMERIZATION STEP OF ACTO.S1 IN THE PRESENCE OF ADP

INTRODUCTION

Studies in chapter 3 have shown that the fluorescence of pyrene labelled actin monitors the isomerization step of the acto.S1 binding complex in the absence of nucleotide. The equilibrium of this step was demonstrated to be sensitive to different experimental conditions, however, varying conditions produced < 5% of the acto.S1 complex in the weakly "attached" state.

Investigations by Geeves et al. (1986) have shown that the fluorescence of the pyrene group monitors this step also in the presence of nucleotide. This allowed Geeves & Jeffries (1988) to characterize the two binding states in the presence of different nucleotides, nucleotide analogues and under different solvent conditions. It was demonstrated in their extensive study that these treatments can be used to reduce the "rigor-like" binding of the acto.S1 complex.

The aim of the experiments reported in this chapter is to examine the influence of ADP and different anions at different ionic strength on the isomerization step. Since both parameters have influence on the equilibrium constant (K_2) of this step, it may, therefore, be possible to use these conditions to weaken the "rigor-like" state further, which would allow for most of the acto.S1 complex to exists in a stable [A-MD] state. In establishing this, it would then allow for structural studies to

CHAPTER 5 BINDING STUDIES OF ACTIN TO S1 BY SLOW TEMPERATURE JUMP

INTRODUCTION

Investigations in chapter 3 have shown that the rate of association of actin to S1 has a very high temperature dependence, and the thermodynamic data of the reaction suggested a conformational change in the proteins. These observations have given the incentive to look at the binding of acto.S1 by the temperature jump method.

Temperature jump perturbation technique for studying chemical and biochemical systems is an established method (Eigen & DeMaeyer, 1963). The standard methods use electrical discharge or laser flash to increase the temperature in a few microseconds and subsequent chemical relaxation of the system to the new equilibrium can be followed between 10^{-6} and 10^{-1} sec (Czerlinski et al., 1964; Czerlinski, 1966). However, since the temperature after the jump is held for not longer than few seconds because of convection and thermal conduction, it prevents observations of longer relaxation times. Slower relaxations have been investigated by switching circulating fluids of two thermostated baths. A device described by Pohl (1968) could equilibrate a spectrophotometer cell within a few seconds. More recently, Nakatani (1985) designed a machine, which pushed a solution at an initial temperature (T_1) through a heat exchanger before entering an observation chamber thermostated at a second temperature (T_2).

In this chapter a simple modification of a stopped flow

CHAPTER 6 THE EFFECT OF HYDROSTATIC PRESSURE ON THE BINDING OF Ca^{2+} TO TROPONIN C

INTRODUCTION

Studies on purified actin and myosin subfragment (S1) presented in chapter 3 have shown that the isomerization step of the acto.S1 complex is pressure-sensitive. This isomerization represents a change from the weakly "attached" to the "rigor-like" complex and has been proposed to be closely related to the force-generating event of the cross-bridge cycle. It has, therefore, been of interest to determine if the pressure-sensitive isomerization identified in solution is also responsible for a pressure-induced change in tension in muscle fibre. Fortune et al. (1989) have investigated the effect of changes in hydrostatic pressure on maximally calcium-activated tension in glycerinated rabbit psoas fibres. These workers reported that steady active tension was depressed by 8% for a 100atm pressure rise. Studies by Brown (1934a;b), and more recently by Geeves & Ranatunga (1990) have shown that tetanic tension in intact muscle fibres is also depressed by 5-8% at high pressure. These findings are consistent with the idea that increased pressure is perturbing force generation in the cross-bridge.

The findings of Fortune et al. (1989) relate a specific pressure sensitive protein isomerization identified in solution to a pressure sensitive transition between two attached states of the active cross-bridge.

In the intact muscle, i.e. no membranes are removed and the

GENERAL DISCUSSION

INTRODUCTION

One aim of muscle biochemistry has been to describe the mechanics of muscle contraction in terms of the kinetics and energetics of the actomyosin interaction. The known energetics of many intermediates in the contractile cycle have been used to construct a biochemically explicit model of the cross-bridge cycle (Eisenberg et al., 1980). Although the mechanism of contraction is not yet understood at molecular level, there has been considerable progress towards this aim in recent years. Studies by Eisenberg & Greene (1980) and Geeves et al. (1984) have suggested a model, in which a myosin head binds weakly to an actin filament, a conformational change in the protein complex occurs, translating the actin filament by 5nm to 10nm, ending in a tight complex between actin and myosin, with myosin forming an acute angle with actin. However, a number of important questions remain unanswered and one of fundamental importance is the nature of the conformational change upon actomyosin binding. One approach that could answer this question is the identification of the state, in which myosin appears to be weakly bound to actin.

Coates et al. (1985) have observed a weakly and a strongly bound acto.S1 state in their pressure jump studies and have shown that temperature, ionic strength and the presence of organic solvent had a marked effect on the two individual steps of the reaction. In this thesis the effect of these parameters have been examined in more detail in order to understand the nature of the reaction in each step. By using the differential effect of

various conditions it was possible to trap some of the acto.S1 complex in the weakly attached state. Stabilizing this state may allow the determination of the acto.S1 structure in solution, which can be compared to the strongly attached state and to structures identified in muscle fibres. Evidence of structural changes in muscle fibre has come from two different conformations observed under non-physiological conditions: a weakly bound state at low ionic strength relaxing solutions and a strongly bound rigor state (Brenner et al., 1982). This idea of two cross-bridge populations is compatiable with many of the structural and mechanical properties of contracting muscle (Huxley & Kress, 1985).

The nature of the structural changes, however, remained undefined. It may involve the actomyosin interface such that the two proteins "roll" past each other, a gross change in shape or size of a single myosin head or "melting" of part of the S2-coiled-coil as suggested by Harrington (1971). In this thesis the thermodynamics of the acto.S1 interaction have been examined in an attempt to ascertain the type of structural changes.

Stabilizing the acto.S1 complex in the weakly attached state

Different solvent conditions were employed in chapter 3 to trap the weakly bound acto.S1 state. Using different monovalent anions at low ionic strength (0.1M) and in the presence of organic solvents showed a negligible effect on the equilibrium constant (K_oK_1) of the weakly bound state. An equilibrium constant of approx 10^5 M^{-1} was observed, which is in agreement with results reported by Coates et al. (1985) for 0.1M

chloride. Increasing the concentration of acetate and possibly of proprionate to 0.5M and the presence of DMSO and ethylene glycol showed no significant change in K_oK_1. This is compatible with observations by Tregear et al. (1984) who have reported that organic solvents have a "stabilizing" effect on acto.S1 in the weakly bound state. However, the presence of chloride ions (and probably bromide and bicarbonate, since these anions gave similar indications) at concentrations well above 0.1M did destabilize the attached state by increasing the rate, at which the two proteins dissociated. There is no direct information to what this effect can be assigned to specifically. It could be due to a change in conformation of actin, S1 or simply in the interaction between the two with no large scale structural change of either species (Harrington, 1971). A clue to these observations is given by Warren et al. (1966) and Stafford (1985) who have reported the same effectiveness of monovalent ions and ionic strength on myosin stability and interpreted their results with the removal of water from the protein.

Electrostatic interactions seem to play an important role in the association of actin and S1 as the affinity of the complex and the rate constant of the association are dependent on ionic strength and solvents. As shown in chapter 3 the association rate constant decreased by a factor of 3-4 on increasing ionic strength and addition of organic solvents. The effect of these parameters on the association rate has previously been reported by White & Taylor (1976) and Konrad & Goody (1982) and was described as an ion-paired charge interaction. However, the

formation of the attached state is a complex reaction and this effect on the formation of a collision complex can not be distinguished from the effect on the rate of transition to the attached state. Yet, these experiments have shown that increases in ionic strength (except for chloride, bromide and bicarbonate) or the addition of organic solvents have very little effect upon the weakly attached state and can hence be used to find conditions where the fraction of attached myosins in the "rigor-like" state can be reduced.

The effect of different experimental conditions on the "rigor-like" acto.S1 state

The effect of monovalent anions at high ionic strength and organic solvents on the acto.S1 isomerization step was also investigated in chapter 3. Using pressure relaxation technique showed that ionic strength at 0.5M and the presence of 40% ethylene glycol or 20% DMSO decreased the equilibrium constant K_2 by a factor of 5 - 6 to a value of approx 20 (table 3.2). Under all these conditions more than 95% of the acto.S1 complex remained in the "rigor-like" complex, which did not allow for structural studies to be carried out of the two states.

Previously, Geeves & Jeffries (1988) have shown that the weakly attached state of the acto.S1 is not affected by ADP but ADP markedly reduces the equilibrium constant K_2 of the isomerization step. In chapter 4 different experimental conditions were used in the presence of nucleotide. Since the fluorescence of pyrene-labelled actin reports the isomerization step specifically (Coates et al., 1985), steady state experiments

were used for direct determination of the fraction of bound actin in the two states. Results in chapter 4 show that the combination of ionic strength and ADP reduced the strongly attached state significantly (K_2 approx 1.6; conditions: 0.5M KCl, 1mM ADP, 20°C, pH 7), leaving approx 60% of the acto.S1 complex in the "rigor-like" state. Complete occupancy of the acto.S1 complex in the attached state by this method was, however, limited by the high protein concentration and the weak binding of actin to S1. The decrease in K_2 with increasing chloride concentration was much larger compared to acetate or proprionate concentration. Replacing chloride with acetate or proprionate in the presence of 1mM ADP showed an approx 30 fold and an approx 5 fold decrease in K_2, respectively. This phenomenon was attributed to a specific chloride effect on the binding of nucleotide to the nucleotide binding site of S1 and was further investigated by stopped flow displacement experiments (chapter 4). Results, however, were inconclusive and a possible answer of the effect of chloride is given by the idea that acetate and proprionate are less effective in removing water from the macromolecules. Previously, Von Hippel & Wong (1962) have shown the degree of hydration of collagen decreases with increasing electrolyte concentration and monovalent anions of the order $CH_3COO^- < Cl^- < Br^-$.

The evidence presented here support the view that major conformational changes occur in the acto.S1 complex and that this conformational change is sensitive to the nucleotide bound to S1. Studies by Eccleston & Bayley (1980) and Shriver & Sykes (1981), using various methods have observed local changes in the

myosin.nucleotide and acto.S1 binding, respectively but no gross structural change of any one species was reported. It may be inferred that the overall conformation in this state is more compact than in the nucleotide-free AM state. Normally proteins, which take up compact conformation are associated with a net increase in weak interactions within the molecule (e.g. hydrogen bonds, van der Waals contact, charge-charge interactions) that contribute to the enthalpy value (Bagshaw & Reed, 1976).

Results in chapter 3 and 4 have shown that the differential effect of ionic strength, solvent and nucleotide can be used to increase the portion of actin bound myosin (S1) in the weak [A-M] state. Although these conditions have not stabilized most of the acto.S1 in the weakly attached state (< 60%), using the different parameters may allow for some studies to be carried out. Future work, e.g. could involve the use of PPi or AMP.PNP in an attempt to completely occupy the acto.S1 complex in the weakly attached state.

Conformational changes associated with the formation of the weakly attached acto.S1 complex

It has been shown in chapter 3 that temperature affects the equilibrium constant K_oK_1 of the weakly attached state and the rate of association K_ok_{+1} markedly. The effect of temperature on the rate of acto.S1 binding has been well documented in the past (Trybus & Taylor, 1980; Marston, 1982; Criddle et al., 1985). Results from experiments in chapter 3 showed that the rate of association of acto.S1 in 0.1M acetate was reduced similarly over a 20°C temperature range as observed in 0.1M chloride by Coates

et al. (1985). Plotting the rate constants against temperature in an Arrhenius graph indicated a break in the slope at approx 10°C (fig. 3.6). The thermodynamic data in table 3.5 showed large differences in entropy below and above the critical temperature, indicating some change in order of this reaction step. Sofar linear Arrhenius plots have been reported for acto.S1 binding in chloride buffer and structural transitions have been suggested because of the large temperature dependence of this reaction (Highsmith, 1977). Konrad & Goody (1982) have regarded the association of actin and myosin (S1) as an entropy-driven process and interpreted the entropy change with a less ordered water structure surrounding the proteins. The results presented here give support to the idea of a structural or phase change of the acto.S1 binding reaction.

The association reaction of actin to S1 was also examined by temperature jump method. A simple modification to a standard thermostated stopped flow machine was carried out, which allowed it to be used as a temperature jump apparatus. This apparatus, which is described in detail in chapter 5, has a sample size of 300ul and can produce temperature jumps both above and below ambient temperature. Temperature jumps larger than 10°C were achieved in less than 150msec, which made it useful for the range of times for acto.S1 binding reactions to be measured. Results from acto.S1 binding experiments in chapter 5 were in good agreement with data achieved in the stopped flow apparatus and in the pressure jump machine by Geeves & Halsall (1986) at 20°C and at 5°C in chapter 3.

Findings in chapter 3 and 5 support the idea that some conformational change occurs in the acto.S1 binding reaction. The high temperature dependence of this reaction, the observation of a breakpoint in the Arrhenius plot and the thermodynamic data in table 3.5 suggest that this process is more complex than a simple binding reaction.

Conformational changes associated with the transition of acto.S1 from the weakly to the "rigor-like" complex

Analysis of the amplitudes of the pressure sensitive step K_2 allowed volume changes to be calculated (table 3.2) to obtain information of the conformational changes and thermodynamics involved. Results in chapter 3 indicated that high ionic strength and organic solvents caused a reduction in volume from approx $100cm^3/mol$ (0.1M KCl) to approx $50cm^3/mol$, which is probably be due to changes in water structure inferred by these parameters. Volume changes of this size imply a substantial rearrangement of the hydration sphere around the protein complex or the interface between the proteins (Gutfreund, 1972; Heremans, 1982). This can be attributed to a conformational change in at least one of the two proteins or the contact area between them and since ionic, polar and hydrophobic groups interact with acto.S1, these have a strong influence on the volume (Kodama, 1985). Hydrophobic interactions are of particular interest as they have a marked influence on the stability when macromolecules are interacting (Von Hippel & Schleich, 1969b). Although little is known about volume changes associated with the hydrophobic interactions. The transfer of non-polar molecules from non-polar

to polar surroundings results in a decrease of molar volume of the solute. It is believed that the hydrophobic hydration is a cooperative phenomenon, in which the exact microstructure of water is very important for the occupied volume. Changes in microstructure when two molecules associate in a hydrophobic interaction, however, is not particularly well understood (Ben-Naim, 1980).

Several pieces of evidence suggest that the myosin head (S1) consists of at least two structural domains and the interaction of these domains appear to be altered upon binding to actin (Vibert, 1988; Vibert & Cohen, 1988). Results in chapter 3 can not give direct evidence that myosin (S1) undergoes a delocalised change of conformation. The source for thermodynamic changes seems to be displacement of water from the surface areas of the two proteins that come in contact during association. Charged and/or hydrophobic side chains are probably involved in the interaction. Borejdo (1983) mapped the hydrophobic sites of myosin and actin and results suggested that either hydrophobic interaction or actin binding are important to induce a shift in the macrostate. Studies by fluorescence resonance energy transfer (FRET) have also reported substantial conformational changes upon actin binding to S1 but have given no direct information on where in the structure of actin or S1 this change takes place (Trayer & Trayer, 1983; Bhandari et al. 1985;).

The effect of different temperatures on the equilibrium constant K_2 of the acto.S1 complex was also investigated in the search for clues of the type of conformational changes involved. The results in chapter 3 from pressure jump experiments (table

3.4) showed that temperature increased K_2 by a factor of approx 2 when decreased from 20°C to 2°C. Calculating the volume change for this temperature range from amplitude changes of the pressure sensitive step K_2 indicated a similar change in volume observed for high ionic strength and solvents (table 3.3). Kinetic methods give no direct information on the nature of the structural change, they do provide thermodynamic data, which can give an indication of the type of structural change. An important parameter for the discussion of pressure-temperature relationship is ΔV. As ΔV is temperature dependent, it also controls the pressure dependence of ΔS through the relationship;

$$d(\Delta G) = -\Delta S x dT + \Delta V dp$$

Using above the equation and from the Maxwell relation, the enthalpic contribution (more negative or less positive) becomes dominant in stabilizing the acto.S1 conformation when the volume changes with increasing temperature. This effect has been reported for other protein-protein interactions (Greaney & Somero, 1979). Paladini & Weber (1981) explained this phenomenon with an increase of free volume or dead space between the associated protein subunits and the trapping of solvent at the interface between proteins. As shown in chapter 3 (ΔH) becomes less positive with an increase in temperature, making (ΔS) less positive at higher temperature. This can be attributed to diminishing water structure and protein conformational change (Eagland, 1975).

Correlation of biochemical and mechanical events

As previously discussed by Coates et al. (1985) and also shown in chapter 3 high pressure reduces the equilibrium constant K_2 of the isomerization step by a factor of 1.5 - 2. If this step is the molecular basis of force generation then a reduction in tension in muscle is predicted. Fortune et al. (1989) have used pressure perturbation method on muscle fibre and observed a depression on maximally Ca^{2+} activated skinned fibres at high pressure. Geeves & Ranatunga (1990) monitored a pressure-induced decrease in tetanic tension of intact fibres. These findings are compatible with the model of Geeves et al. (1984), which suggested that the isomerization is closely linked to the tension generating event in muscle fibre. The change in sensitivity in the fibre system to increases in hydrostatic pressure is consistent with the step identified in solution (Coates et al. (1985); chapter 3). This suggests that hydrostatic pressure is principally perturbing the transition between the attached cross-bridge and a rigor-like tension-bearing state in fibre systems.

If the transition from the "attached" to the "rigor-like" conformation of the acto.S1 is closely linked to the power stroke of the cross-bridge (Geeves et al., 1984) and solvents and high ionic strength reduce K_2 of the isomerization step in solution as demonstrated in chapter 3 then an effect on the tension in muscle is also expected. Previous observations by Gulati & Babu (1982) have demonstrated that an increase in KCl concentration causes a decrease in isometric tension generation in skinned fibres. Clarke et al. (1980) published data that the presence of 50% ethylene glycol decreased rigor tension in insect flight muscle.

These results support the idea that changes identified in the transition step in solution can be correlated to changes in tension generation in muscle fibre.

Results in chapter 6 have indicated that high pressure does not affect calcium binding to troponin C in solution significantly. Under the assumption that the effect of pressure on TnC is no different in muscle fibre, this would imply that the increase in twitch tension observed by Geeves & Ranatunga (1990) upon pressurisation cannot be due to changes in calcium affinity to the regulatory proteins. The augmentation of twitch tension at high pressure can, therefore, be the result of either the release of calcium from the sarcoplasmic reticulum or due to a shift in the inhibitory function of the regulatory proteins. The idea of calcium release from the sarcoplasmic reticulum at 10MPa pressure rise can be tested e.g. by injecting TnC_{DANZ} into muscle fibres. The shift in the inhibitory function of the $(actin)_7Tn/Tm$ unit at high pressure can also be evaluated, using the results presented in chapter 6.

Fortune et al. (1989) and Geeves & Ranatunga (1990) have both observed that pressure induced a decrease in maximally calcium-activated tension in skinned muscle fibre and in tetanic tension in intact muscle fibre, respectively. This effect has been interpreted as the consequence of a specific effect of pressure on cycling cross-bridges. Results presented in chapter 6 have shown that not only the binding of calcium to TnC (< 3%) but also the binding of calcium to EGTA (< 1%) is insignificantly affected by high pressure. This observation would, therefore, allow

pressure perturbation experiments to be performed in skinned muscle fibre at low calcium concentration, using EGTA buffers with no perturbation of the free[Ca^{2+}]. If, under these conditions pressure perturbation causes a tension increase, then this is likely be due to a shift in the inhibitory function of the regulatory proteins.

CONCLUSION

Although the experimental approach of the study of purified acto.S1 in solution compared to muscle fibres is different, the correlation of biochemical findings to *in vivo* observations gives support of the two step binding model proposed by Geeves *et al.* (1984). It has been shown that conformational changes occur upon the binding of acto.S1 to form the weakly attached state and that the transition between this state and the "rigor-like" state involves structural changes sufficient to account for the force generating event. The various experimental conditions, which influence the transition in solution also affect force generation. However, for this transition to be fully identified as the force generating event, it must be coupled with the ATP product release. Solution studies have given little direct information sofar on the acto.S1 ADP.Pi state as a result of minimal binding of phosphate to the complex (Sleep & Hutton, 1980). Isotope exchange experiments have provided a method of monitoring this step in solution and sofar limited information on an intact system has been obtained (Webb *et al.*, 1986). Correlation of these findings in solution and intact muscle are the ultimate test of this model.

The potential of pressure relaxation method for the study of acto.S1 in solution has been demonstrated in this work and its application to measure cross-bridge events in muscle fibre has been referred to. This perturbation method not only provided information about rates of acto.S1 interaction but also gave indications of volumetric behaviour of these proteins. From the concentration dependence of the specific volume, information was obtained on the protein-protein interactions. The amplitudes of displacement from equilibria at different reactant concentrations and at different temperature changes were used to calculate equilibrium constants and thermodynamic parameters, respectively, which provided clues of structural changes. All this information proved valuable in the elucidation of the acto.S1 binding mechanism and make this method a powerful tool. Since the understanding of mechanical force on molecular level remains a major aim of muscle research, perturbation method used on isolated proteins in solution and on muscle fibres can, therefore, make a valuable contribution towards this aim. Using this technique, several challenging questions to date could be addressed and results could be correlated with findings in muscle fibres; e.g. the cooperative behaviour of the $(actin)_7$Tn/Tm unit in the presence of calcium. The elucidation of the overall mechanism could give an answer, at which step Tm/Tn affects the actin binding, ATPase rate and subsequent Pi-release; or the possibility of the cooperative binding of the two heads of S1 (HMM) to actin. The difference in behaviour of the two heads in the kinetic ATP-cycle could have significant consequences on

isometric tension in muscle fibre.

Having shown in this thesis that conformational changes do occur upon binding of actin to S1 to form the weakly attached state followed by changes to form the rigor-like state, future work could involve e.g the use of rapid-freeze-etching methods to observe these conformational changes in a high resolution electron microscope. Although the different experimental treatments have not achieved complete occupation of the weakly attached state, using this method could allow for studies of the two interacting proteins to be carried out and results could be compared with published data on whole muscle fibre.