Biochemical Approach to Structural Changes in the Contractile Protein Myosin

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Zusammenfassung

Im ersten Teil wird eine Übersicht über den heutigen Stand des Wissens über den Kontraktionsvorgang und seine Steuerung auf molekularer Ebene gegeben. Da die kontraktilen Eiweisse Myosin und Actin nicht nur in Muskeln, sondern auch in vielen andern nichtmuskulären Zelltypen gefunden wurden, ist anzunehmen, dass der Bewegungsvorgang in allen eukaryoten Zellen auf dem gleichen molekularen Mechanismus beruht. Nach der Besprechung der molekularen Eigenschaften der kontraktilen Eiweisse, ihrem Aufbau in den Filamenten des Sarcomers der Muskelzellen und ihrer zyklisch ablaufenden Wechselwirkungen, die zur Kontraktion führen, wird die Frage der Umwandlung von chemischer in mechanische Energie behandelt. Gewonnen wird die für den Bewegungsvorgang nötige Energie aus der enzymatischen Spaltung des in allen Zellen vorhandenen Adenosintriphosphats (ATP). Nach neusten Erkenntnissen ist die Umwandlung der aus der Spaltung von ATP gewonnenen chemischen Energie in mechanische Bewegung mit einer Reihe von nacheinander ablaufenden Konformationsänderungen in den kontraktilen Eiweissen, d. h. Veränderungen der räumlichen Anordnung der Eiweisse und ihrer Untereinheiten zueinander, verbunden. Diese Mikrobewegungen auf molekularer Ebene, die zwischen den Kopfteilen der stäbchenförmigen Myosinmoleküle und dem Actin ablaufen, sind letztlich für die makroskopischen Muskelbewegungen verantwortlich. Gesteuert werden diese molekularen Mikrobewegungen durch Calciumionen. Calcium stellt das letzte Glied in der Kette der Impulsübertragung, die vom Zentralnervensystem bis zum kontraktilen Apparat in den Muskelfasern führt, dar.

Im zweiten Teil wird über eigene Arbeiten berichtet, die zum Ziel haben, mit biochemischen Methoden solche mit der Spaltung von ATP einhergehenden Konformationsänderungen des Myosins, welches gleichzeitig neben seiner Bewegungsfunktion auch enzymatische Eigenschaften besitzt, nachzuweisen. Es ist möglich, solche Unterschiede aufgrund der veränderlichen Reaktionsfähigkeit von Seitengruppen bestimmter Aminosäuren im Molekül gegenüber gewissen Reagenzien festzustellen. Durch Gebrauch radioaktiver Alkylierungsmittel konnten so mehrere Sulfhydryl-

gruppen im Myosin markiert und nach proteolytischer Verdauung zu Fragmenten in verschiedenen Teilen dieses grossen Moleküls lokalisiert werden. Die Resultate lassen erkennen, dass in den Regionen, in denen Bewegungen einzelner Teile des Myosins gegeneinander zu erwarten sind, tatsächlich enzymatisch bedingte Konformationsänderungen auftreten, die Veränderungen der Reaktionsfähigkeit der dort sitzenden Sulfhydrylgruppen bewirken. Mit dieser Methodik können kleinste Formänderungen in den einzelnen Untereinheiten des Myosinmoleküls wahrgenommen werden, wie es zurzeit weder mit der Elektronenmikroskopie noch der Röntgendiffraktion möglich ist bei diesem Eiweiss.

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Abbreviations

AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	ATP phosphohydrolase EC 3.6.1.3
APPNP	5′-adenylylimidodiphosphate
ATPγS	adenosine-5′-0-(3-thiotriphosphate)
HC	heavy chain
LC	light chain
IAA	iodoacetamide
NEM	N-ethylmaleimide
P_i	inorganic phosphate
PP	pyrophosphate
SDS	sodium dodecyl sulfate
DEAE-Sephadex	diethylaminoethyl-Sephadex
Tris	Tris-(hydroxymethyl-)aminomethane

A. Molecular Basis of Motility (Review)

1. Contractile Proteins

Contractility is one of the fundamental characteristics of cells from higher organisms and of all eukaryotic cells. Life is dependent not only on directional movement of the whole organism but equally on the motility of each single cell in order to maintain its biological functions including processes such as cell division, preserving the shape of the cells and different types of intracellular movement, to mention but a few. It has become apparent that close similarities exist in a number of instances between the proteins involved in contraction in striated muscle, and proteins present in non-muscle cells in which movement occurs. On the basis of protein composition 2 classes of motile systems can be distinguished, the eukaryote flagellum and the actomyosin system of muscles and cytoplasmic streaming. If we are to understand the functioning organism we must understand the cell and in turn to comprehend the living cell we must isolate the individual molecules and study their properties and interactions quantitatively. Ultimately when we understand the biochemistry of the individual constituents in molecular terms, one may undertake to reassemble the total system.

Thanks to the well ordered fashion in which the contractile proteins are built up in muscle the combined efforts of structuralists, biochemists and mechanists has made it possible to gain a rather detailed insight into the molecular mechanism of contraction and its regulation. The almost crystalline myofibrils, composed of parallel filaments in segmented arrays, first caught the eye of light microscopists and later provided material for electron microscopy and X-ray diffraction. Evidence is accumulating, however, that on the molecular basis, the same principle of movement applies also to non-muscle cells where actomyosin-like proteins have been found, although its regulatory mechanism may vary from one cell type to another. More recent findings indicate that even in the motile system associated with cilia and flagella which consists of a different type of contractile proteins, tubulin and dynein, the same molecular principle seems to be maintained for generation of movement as in muscle, i.e. the sliding of filamentous structures past each other, driven by repetitive interactions of cross-bridges between them [1–3]. A further similarity between the two different contractile systems seems to be that, whatever type of additional regulatory proteins are present, these are able to interact with Ca ions and hence allow the motility to be monitored by changes in the intracellular free Ca ion concentration [3–5].

It is well established that in striated muscle the contractile proteins, myosin and actin, are localized in distinct filaments in the sarcomere, the contractile unit, of the myofibril. These myofibrils containing a large number of sarcomeres in series are long thin structures usually about one μm in diameter, and lie densely packed in the muscle fibre or cell which itself has a diameter of about 0.1 mm. The parallel alignment of the myofibrils in the muscle cell gives rise to the striation pattern, but the details of the structures of the sarcomeres with a length of around 2 μm cannot be resolved in the light microscope.

Actin in its monomeric form, also called G-actin, is a globular protein with a diameter of 5.5 nm consisting of one polypetide chain with a molecular weight of

41,785 dalton and whose amino acid sequence is known [6]. Comparative amino acid sequence studies [7, 8] and peptide maps [9] of actin from various sources including non-muscular contractile systems have shown that, as one of the most conservative proteins, it has preserved its primary structure during evolution. In the presence of mono- or divalent cations G-actin polymerizes into F-actin forming up to 10 μm long filaments with molecular weights in the order of several millions. Such a filament consists of 2 actin strands arranged in a double right-handed helical array with a helical repeat distance of 36–37 nm [10, 11].

In its polymerized F-form actin contains tightly bound ADP and Ca, one mole of each per mole of actin. It seems that the chief function of the bound metal and nucleotide is to stabilize the structure of the actin molecule rather than to participate directly in interaction with myosin or with other actin molecules [12]. In muscle the actin filament of a length of about 1 μm per half sarcomere has the same structure (fig. 1).

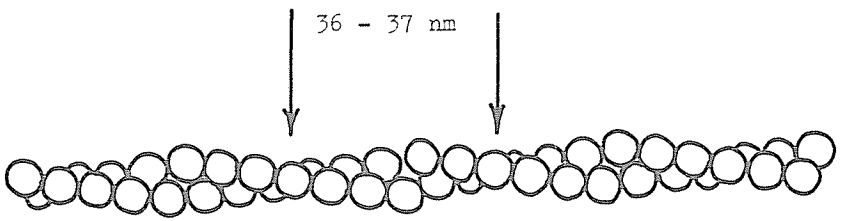


Fig. 1. Actin filament. Arrows indicate crossover points of the two strands coiled around each other.

Myosin plays a key role in muscle contraction serving, at the same time, as a structural protein as well as an enzyme catalyzing the hydrolysis of ATP into ADP and inorganic phosphate. It amounts to about 50% of the myofibrillar proteins [13–15] and is among the largest proteins whose structure has been well characterized. It is a highly asymmetric molecule with a length of about 140 nm and a molecular weight of 470,000 dalton [16]. It consists of 6 subunits, 2 heavy chains (HC) with molecular weight of around 200,000 dalton each and 4 light chains (LC) in the 20,000 molecular weight range [17, 18]. The 2 heavy chains form the alpha-helical tail structure and, on one side, end up in a globular head portion each, to which are attached the 4 light chains (fig. 2). Myosins from slow-contracting skeletal muscles and from heart [17, 19] as well as from smooth muscle cells [20, 21] all contain 2 types of light chains, probably 2 moles of each per mole of myosin, which can be separated on the basis of their difference in molecular weight. This seems also to hold for myosins isolated from non-muscle cells [22, 23] including from blood platelets [24]. In fast-contracting muscles the situation is more complex as myosin from such muscles invariably contains 3 types of light chains with different molecular weights. Studies on stoichiometric subunit distribution indicated that each myosin contained 2 moles of LC-2 with a molecular weight of 19,000 dalton but apparently somewhat more than one mole of LC-1 (molecular weight = 21,000 dalton) and on the other hand less than one mole of LC-3 (molecular weight = 16,500) [19, 25, 26]. Recent reports indicate that this uneven subunit distribution may arise from the coexistence of different amounts of 2 types of

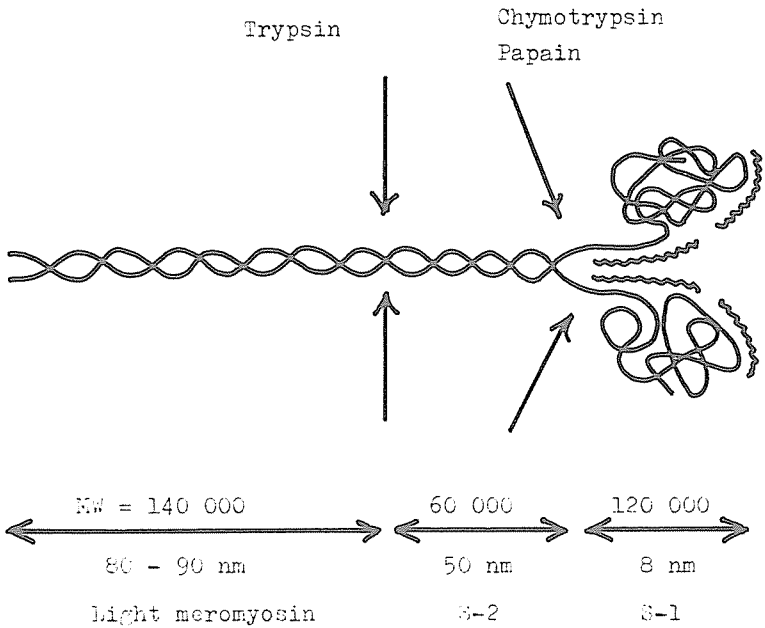


Fig. 2. Subunit composition of the myosin molecule. Arrows indicate regions of proteolytic cleavage producing the insoluble tail (light meromyosin), the soluble neck portion (S-2) and the two head portions (S-1). Associated to the heads are the four light chains.

homodimeric myosin isoenzymes, one containing 2 LC-1 the other 2 LC-3 beside the couple of LC-2 [27, 28].

Limited proteolytic digestion under mild conditions with trypsin, chymotrypsin or papain has allowed the large myosin molecule to be dissected into smaller portions in such a way that the resulting fragments have preserved their biological functions [29–31]. Intact myosin is only soluble in solutions of high salt concentrations (ionic strength above 0.4) and becomes insoluble at an ionic strength less than 0.25. Under appropriate conditions with respect to ion composition and pH, myosin forms in vitro aggregates which resemble very much the natural myosin filaments found in the muscle sarcomere [32]. Molecules that point in opposite directions are bonded together at the centre of the myosin filament, while growth proceeds by addition of more molecules to both ends (fig. 3). The backbone of the filament consists of the interacting myosin tails (light meromyosin) which form a regular helical pattern with probably 3 molecules in register at each level [33]. The neck region of the molecule, S-2, which when isolated, is soluble at low ionic strength, does not remain attached to the filament shaft and may serve as a hinge to permit the head portions of the molecules to project out from the myosin filament and swing over to reach the actin filament when so required. The 2 head units each containing an ATPase centre and an actin-binding region form the so-called cross-bridges seen in the electron microscope between the myosin and actin filaments. According to the assembly of the myosin mole-

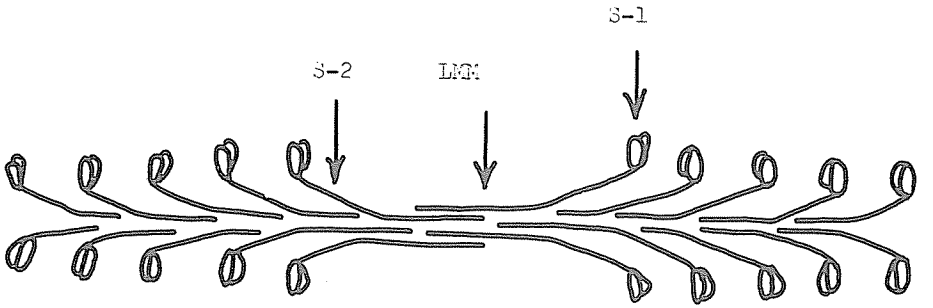


Fig. 3. Myosin filament demonstrating the projecting head portions of the molecules as well as their polar assembly (LMM = light meromyosin; S-1 = heads; S-2 = neck region).

cules in the filament shaft, the projections of these cross-bridges occur at sections 14.3 nm apart arranged in a helical fashion with a pitch of 43 nm.

Unlike actin, myosin from different sources exhibits different properties with respect to solubility, polymerization into filaments, its subunit composition and enzymatic properties. These variations are not only due to the fact that different types of myosin contain different sets of light chains which are thought to influence mainly its interaction with actin [34–37], but also arise from association with additional proteins such as M- and C-proteins e.g. in the case of striated muscle [38, 39]. The myosin of blood platelets and cultured fibroblasts e.g. seems to be more soluble than muscle myosin, it does not aggregate readily into filaments at physiological ionic strength and cannot be sedimented at high centrifugal force [40]. Furthermore, while the actin content in different motile cells is first of all substantial, i.e. around 15% of total protein, and secondly rather constant, varying only by a factor of 2 between striated muscle (= 19%) [15] and e.g. blood platelets (= 10%) [41, 42], the content of myosin differs by a factor of over 100 between different systems (table 1). This makes it much more difficult to detect myosin in some cell types either by microscopic or biochemical methods. All myosins so far isolated from different sources consist of both heavy chains and light chains, display the same highly asymmetrical rod-like shape bearing 2 globular head parts at one end [48], catalyse the hydrolysis of ATP and are able to interact with actin, indicating that they may be the general energy transducing enzymes for cell motility.

Table 1. Myosin content and molar ratio of actin to myosin in different motile cells

Cell type	% Myosin of total protein	Molar ratio of actin to myosin	References
Insect flight muscle	40	3	[43, 44]
Skeletal muscle	35	6–7	[15, 44]
Vertebrate smooth muscle / Taenia coli smooth muscle	3–5	30	[44, 45, 46]
Blood platelets	1	100	[42]
Acanthamoeba	0.3	200	[47]

2. Actomyosin Interaction

In view of the separate localization of actin and myosin in distinct filaments the interaction of these proteins, as the underlying process of muscle contraction seems to be required in order to produce a directional force or to resist being stretched. Only such an interaction would allow a continuous structure to be built up, running along the myofibril by linking all the sarcomeres together via the actin filaments which in turn are attached to the Z-lines. From biochemical studies it is known that the ATPase activity of myosin on its own, under conditions of the muscle at rest, is low, but that it can be greatly activated by the presence of actin. On the other hand, there is no case where such a specific protein-protein interaction has been shown to take place at long-range, in the absence of a direct physical combination (if only a transitory one) between the molecules concerned [49].

Isolated actin and myosin do in fact combine very readily in solution to give the so-called actomyosin complex. The tight combination in the absence of nucleotides occurs at high salt concentration where the proteins are dissolved as well as at low ionic strength where they precipitate, indicating that this interaction is mainly governed by hydrophobic attraction forces. Thereby both heads of a single myosin molecule may combine each with a different actin monomer [50, 51]. That both myosin heads are able to bind simultaneously with the actin filament may be demonstrated by the fact that those cysteinyl thiol groups associated with the active centre of each head and easily blocked in isolated myosin, are fully protected from reaction with alkylating reagents when myosin interacts with actin [52]. X-ray studies [53, 54] and electron microscopy [55] on ATP-depleted muscle indicate that in rigor when the muscle is stiff and cannot be stretched, the myosin cross-bridges are in an angled position and seem to reach over to the actin filaments.

In a living muscle at rest, in the presence of Mg-ATP, however, the cross-bridges are drawn back toward the shaft of the myosin filaments and adopt a rectangular position. The muscle can now easily be stretched without resistance. Addition of Mg-nucleotides or Mg-pyrophosphate to an actomyosin solution at high ionic strength leads to dissociation of the 2 proteins which now may be physically separated by centrifugation and the formerly protected thiol groups of myosin heads become reactive again. It therefore seems overwhelmingly probable that a direct mechanical attachment of the cross-bridges which represent the active head portions of the myosin molecule, to the actin filaments occurs during the splitting of ATP in muscle contraction. Given that cross-bridge attachment takes place, this link provides the most likely site where the mechanical force for contraction may develop.

3. Contraction Mechanism

Thanks to the repeating periodicities of the order of a few tens of nm characteristic for the 2 types of filaments which are detectable by low angle X-ray diffraction techniques, it was possible to reveal the precise hexagonal array of the actin and myosin filaments within the sarcomere. The filaments are held approximately 40 nm apart from each other by long-range electrical repulsive forces as has been discussed by

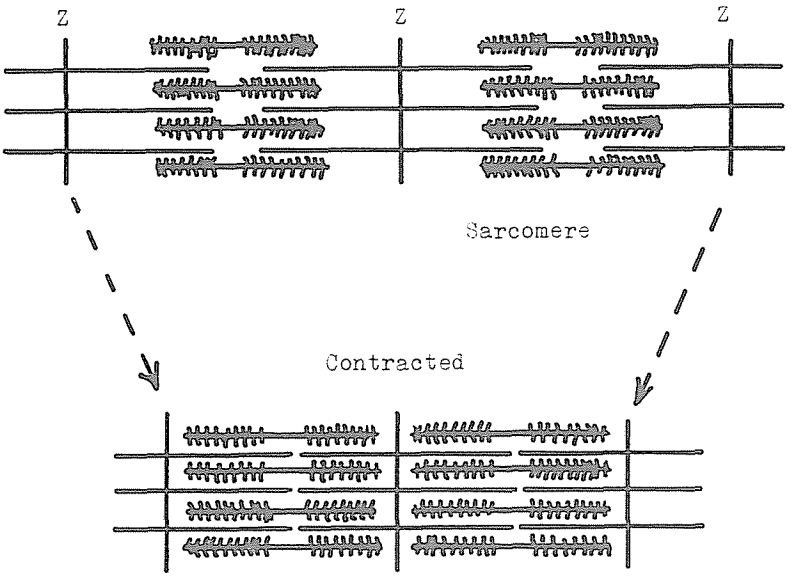


Fig. 4. Assembly of actin and myosin filaments in the sarcomere of the myofibril.

ELLIOTT [56]. The myofibrils are then built up of a long succession of partially overlapping, interdigitating arrays of longitudinal protein filaments [57]. The sliding filament model of contraction was put forward independently in 1954 by H. E. HUXLEY and HANSON [58] and A. F. HUXLEY and NIEDERGERKE [59] once it had been recognized that during contraction when the sarcomeres shorten, the length of the 2 types of filaments remains constant and their characteristic repeat sequences do not change (fig. 4).

A myosin filament has a length of about 1 μm and is composed of 250–300 myosin molecules [60]. These molecules are assembled in the filament in such a way that in the rigor state all the head portions, or cross-bridges, which reach over to the actin filament, are in an angled position, pointing back toward the sarcomere centre from both ends of the myosin filament. This polar arrangement would ensure that the contribution from each cross-bridge to the force generation will add up in the proper direction. According to the packing of the 2 types of filaments in the hexagonal array, each myosin filament is in both halves of the sarcomere surrounded by 6 actin filaments such that each actin filament is able to interact with cross-bridges projecting from 3 different myosin filaments. If an actin filament has a length of about 600–700 nm one may calculate from the X-ray data on the filament periodicity and the dimension of a single actin globule that it must contain 250–300 actin monomers. Taking both sets of actin filaments on either sarcomere side into account, this adds up to a ratio of actin to myosin molecules of 6–7 to one, a number in agreement with that deduced from biochemical studies on skeletal muscle given in table 1.

In resting muscle the cross-bridges are not attached to actin, the filaments can slide past each other under external force, the muscle is plastic and extensible. When it contracts the system may work on the assumption that those cross-bridges which are

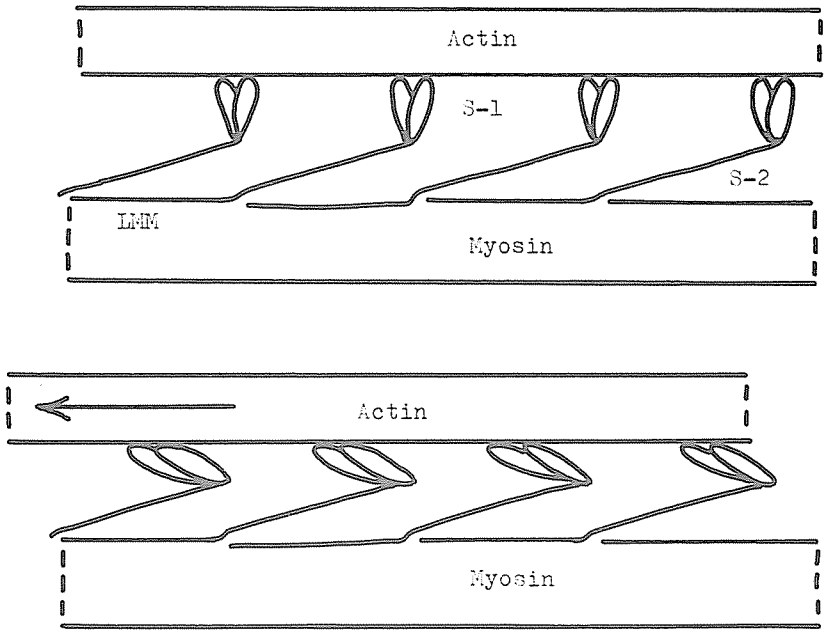


Fig. 5. Assumed contraction mechanism. Directional movement may be brought about by rotation of the myosin head portions (S-1) around their flexible connection with the neck region (S-2) of the molecule.

located in that part of the myosin filament which overlaps with the actin filaments, undergo interaction with actin in a rectangular position. This may then be followed by some conformational changes in the myosin head portions leading to a tilting movement of the cross-bridges which, anchored now on the actin molecules, must necessarily result in a relative movement of the 2 types of filaments past each other (fig. 5). The close relationship of tension development of the muscle to its sarcomere length supports the idea that the cross-bridges act as independent force generators in the overlap region of the filaments [61, 62]. The force generation is thought to be produced by the tendency of the attached cross-bridge to move through a series of positions of progressively lower potential energy [63]. By this rotation, exerted by the myosin head portions, the relative movement of the 2 filamentous systems covers a distance of 7–8 nm or so.

If the actin filament in both halves of a sarcomere would be pulled towards the sarcomere centre by one rotational power stroke of attached cross-bridges, and if that happens in all successive sarcomeres, the muscle would only shorten by about 1% or less. However, since a muscle can shorten considerably more than that, the cross-bridges must turn over many times during a contraction. The attachment would then break away at the end of the first movement enabling the cross-bridge to return to its initial rectangular conformation in preparation for its next cycle of action. The cross-bridges perform their cyclic movements independently of each other so that at any

given moment of contraction a variable number which may well be below 50%, is to be found attached [54]. The combined effect of all cross-bridges undergoing these asynchronous cycles of attachment, pulling and detachment, produces a steady sliding force as long as the muscle is active. Considering a 5% shortening of a fast-contracting skeletal muscle takes place in a time span of about 10 msec then, one would have to assume that the cross-bridges are performing their rowing power strokes at a frequency of several 100 per sec as long as the muscle is activated. The macroscopic property then is the summation of the molecular processes occurring at the single myosin cross-bridges. On the other hand, experimental evidence indicates that, e.g. in fibrillar insect flight muscles, synchronized beating of cross-bridges is able to produce oscillatory movements of about only 1% length changes but with a frequency of over 1000 per sec [64]. In this system in contrast, the cross-bridges are all beating synchronously and never let go their point of attachment on the actin filament.

Since myosin ATPase activity is high only when the heads interact with actin and since they do so in muscle only during the force generating cycle of the cross-bridges, the splitting of ATP will automatically be linked to the external work performed by the muscle. Thus there seems to exist a very tight coupling on the molecular level between the work output and the chemical process supplying the energy required for it.

4. Mechano-Chemical Coupling

The transduction of energy by biological systems seems to require some form of subcellular fibrous or membranous structures [65]. This fact poses obvious difficulties in elucidating the energy converting step or steps from observations made on extracted proteins, since the method of preparation does not usually leave the structures intact. A further difficulty is that the structure must impose some constraint on the enzyme system in vivo to ensure that the energy involved is converted in a biologically useful way and not wasted via alternative pathways. As KORETZ et al. (1972) [66] have stressed for the particular case of muscle contraction, the high efficiency with which contracting systems convert chemical into mechanical energy must be due in some degree to the aggregated states of actin and myosin and the relative positioning of the filaments in an overall structure. Frog muscle converts energy at an estimated 50–60% efficiency from a comparison of work output with creatine phosphate and ATP hydrolysis [67].

Perhaps the oldest accepted concept is that energy transduction proceeds via some form of energy rich phosphate bond. However recent results in the field of oxidative phosphorylation in both mitochondria and chloroplasts, as well as ATP hydrolysis by myosin and actomyosin, indicate that this concept should be discarded since the search for phosphorylated intermediates has proved unsuccessful in all cases [68]. In fact in all cases the step ATP --- ADP plus P_i seems to be reversible so that there must be some other step giving direction and control to the transduction process. It has been suggested that protein conformational changes coupled with ATP and ADP binding and release play the main role in transferring energy [69–71]. On the other hand, physiological experiments on intact and glycerinated muscles also yield strong evidence that some process other than the splitting of ATP is responsible for the

mechano-chemical coupling. When contraction is reversed, i.e. muscle is stretched under activating conditions so that work is done on the muscle, there is no resultant synthesis of ATP [72–74]. This implies that the mechano-chemical event is cyclic and unidirectional and is not an equilibrium which may be moved in either direction.

Although the force generating step involves an interaction of the myosin cross-bridges with the actin filament, some insight into the energetics of the process is obtained from a study of the ATP hydrolytic cycle of myosin alone. Most attention has been paid to the isolated head portion of the molecule for which this cycle comprises 7 elementary steps according to BAGSHAW and TRENTHAM [75]:

$$M + ATP \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} MATP \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} M^*ATP \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} M^{**}ADPP_i \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} M^*ADPP_i$$
$$\underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} M^*ADP + P_i \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} MADP \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} M + ADP.$$

From the values of the rate constants associated with each step it can be calculated that the protein-nucleotide complex spends most of the time (above 95%) required for one single cycle in the forms M^*ATP and $M^{**}ADPP_i$ because the constants k_{-2} and k_{+4} which move the enzyme backwards or forwards away from this equilibrium, are very small (of the order of 0.02 sec^{-1}). The myosin intermediate species whose conformations have been distinguished from one another by various techniques are denoted by asterisks in the kinetic scheme. If the kinetic pathway followed in vivo is similar, and there is evidence that the hydrolysis of ATP by myosin in its filamentous gel state does proceed by the same steps [76], then we may draw some important consequences relating to contraction (fig. 6):

A. In the active state the myosin cross-bridges interact with actin and the hydrolytic rate is enhanced some 2000 fold, thus actin must have its modifying effect on

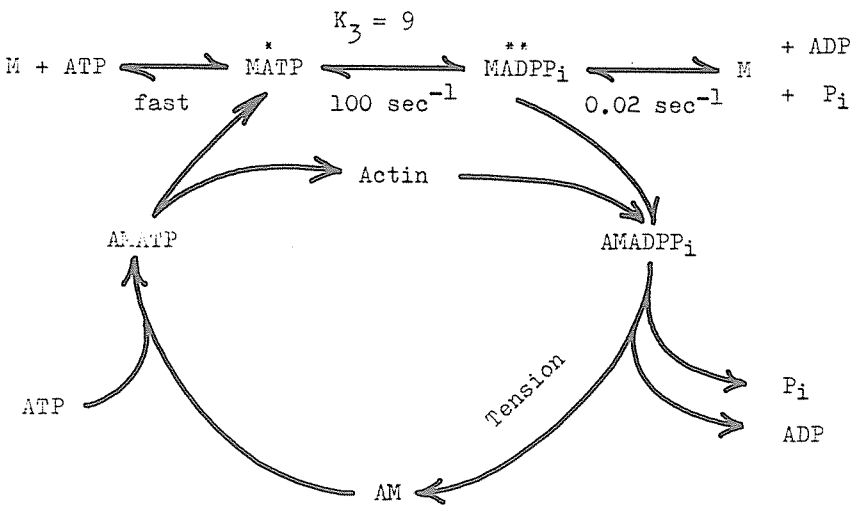


Fig. 6. Simplified kinetic scheme of myosin ATPase. Arrows in the circle follow the actomyosin cycle. During actomyosin interaction development of tension in the cross-bridges is supposed to occur in conjunction with release of the products. M = myosin.

the myosin ATPase primarily by intervening at step 4 and accelerating the rate of product release, as indicated in fig. 6.

B. The low value of the equilibrium constant K_3 indicates that the free energy furnished by the splitting step is about 5 kJoule/mole only. However, as mentioned above, muscle converts chemical into mechanical energy with high efficiency and since about 30 kJoule/mole of free energy is released by the hydrolysis of ATP overall [77], the actual splitting step cannot be responsible for delivering the energy important for force generation.

C. It is most likely that the binding of the substrate ATP as well as the desorption of the products ADP and P_i together with the ensuing conformational changes are the energetically important steps during contraction.

It was earlier suggested that the binding of ATP to actomyosin should supply sufficient energy to dissociate the strong rigor link [78]. These observations support the original proposal for the sequence of steps during one contraction cycle of LYMN and TAYLOR in 1971 [79], who concluded that actomyosin dissociation occurs prior to ATP hydrolysis, which consequently occurs on myosin alone and that actin then reassociates with the myosin-product complex. One therefore expects that it is during the ensuing actomyosin interaction, involving the release of the products, that the transformation of chemical into mechanical energy occurs. The energy is used up in the production of tension in the cross-bridge and the cycle thus ends in a state of low energy with the 2 proteins bound tightly in the rigor state. The cycle then restarts upon binding of the substrate whereby the cross-bridge returns to its energized state, ready to interact again.

5. Regulation of Contraction

The cell of the fast-contracting skeletal muscles represents the most highly differentiated motile system. When the activating stimulus reaches such a cell it attains its state of full activity within a couple of msec. During this time interval the turnover of metabolic energy and the rate of ATP splitting increase by a factor of about 2000 [80]. This figure immediately demonstrates the need for an extremely sensitive and reliable mechanism of regulation.

The intracellular Mg ion concentration remains high (i.e. in the millimolar range) at all times whether the cell is in the active or resting state. These ions are required not only for the activated actomyosin ATPase occurring during contraction, but also for the dissociation of actomyosin in relaxation. Myosin can in fact hydrolyse ATP without Mg ions if monovalent cations e.g. K ions are present, but in this case the kinetic pathway does not contain the long-lasting myosin-nucleotide complex required during relaxation [76, 81] and the rate of hydrolysis is high which would lead to a wastage of ATP. On the other hand the intracellular Ca ion concentration is maintained at a very low level, below 10^{-7} M free ion concentration, during relaxation. This is achieved by active pumping of these ions out of the cytoplasmic medium by various membrane systems such as the cell membrane itself, mitochondria and the sarcoplasmic reticulum.

The role of Ca ions in muscle cells in general is that of the last transmitter in the

chain of events originating in the central nervous system. Upon depolarisation of the membranes there is a Ca ion release into the cytoplasm. This movement of ions has as its target the Ca-receptor regulatory proteins thus triggering various processes such as secretion, contraction and metabolism. In muscle cells in particular, the switching on of the glycogen catabolic pathway by Ca ions [82] therefore occurs simultaneously with the onset of contraction. This ensures the maintenance of a high concentration of ATP which would otherwise be depleted within seconds of fully active contraction.

To date, a wide variety of Ca-receptor proteins have been found [82] all having molecular weights in the 10,000 to 20,000 range. These associated with the contractile apparatus of the myofibril may reside either on the actin or myosin filament [83] depending on the animal species. Nevertheless, wherever their localization, comparison of their respective amino acid sequences indicates that all have evolved from a common ancestoral peptide [84, 85]. The presence of these Ca-binding regulatory components indicates that the Ca ions do not act directly on the pure actomyosin system. In fact, the removal of Ca ions from a milieu containing actin, myosin, ATP and Mg ions alone causes neither a significant reduction in the rate of ATP hydrolysis nor a dissociation of the actomyosin complex [86], both of which are known to occur under conditions of relaxation in muscle.

In the case of skeletal muscle, regulation results from conformational changes which take place on the actin filament in a cooperative way [87]. It is achieved by the interplay between the components of the so-called regulatory protein complex which (for the transition from resting to active) is initiated by the binding of Ca ions to one of the 3 troponin components and ends with the physical displacement of tropomyosin. The primary structure of all 4 regulatory protein components troponin-C [88], troponin-I [89], troponin-T [90] and tropomyosin [91], is now known and this has allowed the recognition of those peptide sequences which are involved in the subtle protein-protein interactions. Their arrangement on the actin filament is illustrated in fig. 7. Tropomyosin consists of 2 alpha-helical chains coiled around each other, both having a molecular weight of 33,000. This rod-like protein has a length of about 40 nm [93].

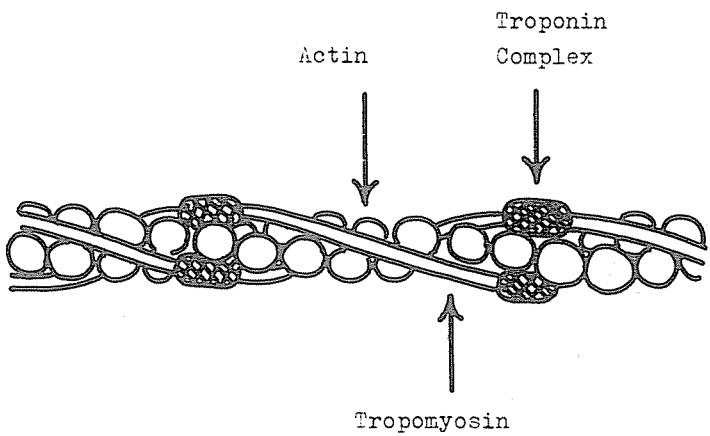


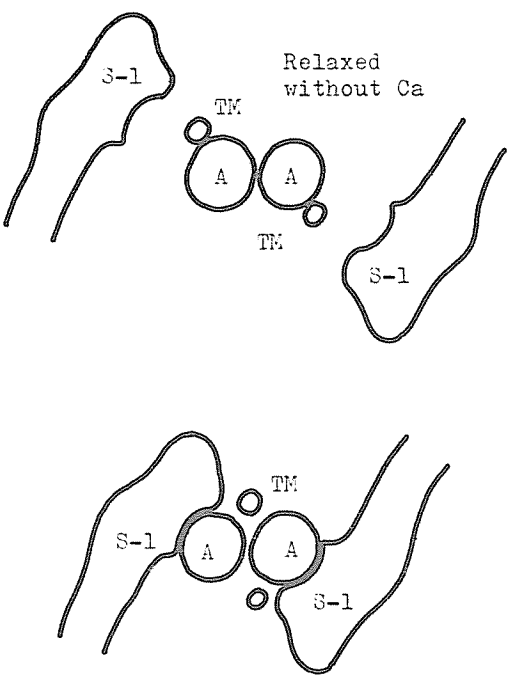
Fig. 7. Localization of the regulatory proteins in the actin filament (after EBASHI and ENDO, 1968) [92].

It appears to be bound to actin, lying in the grooves formed by the coiling of the 2 actin strands, each molecule extending over a length of just one pitch of the actin filament repeat. The 3 components of troponin are associated together and bind to the actin-tropomyosin filament at intervals of 38.5 nm [94]. This ordered constellation of subunits within the filament implies that natural stoichiometric relationships are to be found between these proteins of the myofibril (table 2). Following the binding of Ca ions to troponin, the tropomyosin molecules move a distance of 1–2 nm deeper into the grooves of the actin double strand [95, 96]. This mechanical displacement removes the steric hindrance which prevents the myosin cross-bridges from interacting with actin in the absence of Ca ions (fig. 8).

Table 2. Protein composition of rabbit skeletal muscle myofibrils

Protein	Molecular weight	% of total protein*	Mole per mole myosin	Role
Myosin	470,000	47	1	Contraction
Actin	41,785	28	7	Contraction
Tropomyosin	66,000	6.3	1	Regulation
Troponin-T	30,503	3.1	1	Regulation
Troponin-I	20,897	2.0	1	Regulation
Troponin-C	17,846	1.6	1	Regulation
Others		12		Structure (?)

* In part after POTTER (1974) [15].



Active muscle in presence of Ca

Fig. 8. Steric configuration of tropomyosin and myosin heads relative to the cross-sectioned actin strands in relaxed and Ca-activated muscle (after HUXLEY, 1972) [96].

The Ca ions are actively pumped back into the membrane system of the sarcoplasmic reticulum when the nerve stimulus ceases and the steric hindrance becomes operative again lowering the affinity of the product-loaded cross-bridges for actin. It is an important fact that only when a nucleotide is bound to myosin, does the affinity to actin change with the tropomyosin movement. Muscles in rigor do not respond to the removal of Ca ions. Biochemical results have also shown that the presence of a nucleotide is required for regulation [87, 97]. Thus, under relaxing conditions, the myosin molecule retains the products of hydrolysis ADP and P_i, poised in an intermediate stage of the overall hydrolytic cycle, ready to interact with actin again. This state of myosin in the relaxed muscle keeps the ATP to ADP plus P_i far to the left of the equilibrium thus ensuring that the concentration of the substrate remains always high.

In molluscan muscle it is known that regulation is mediated by changes on the myosin filament [98, 99]. Although these changes are initiated by the binding of Ca ions to one type of subunits of myosin, it may be assumed that the mechanism of regulation is similar in principle to that described above, since both these systems contain tropomyosin associated with actin. In both systems the tropomyosin is thought to mediate myosin-actin interaction by changing its position on the actin filament depending on the Ca ion concentration [100]. In quite a number of species of the animal kingdom there is a combination of both types of regulatory systems to be found [83].

In addition to the highly specialized rapid control mechanism of interaction of actin with the myosin cross-bridges by Ca ions which act as allosteric effectors, regulation of movement is further governed by subtle feed-back processes involving covalent protein modification. The reversible phosphorylation at specific sites on one type of the myosin light chains (in myosins from fast, slow and heart muscles it is the LC with a molecular weight of 19,000 dalton), on troponin-I and troponin-T components and on tropomyosin of the regulatory protein complex represents an intricate background modulation rendering the contractile system more or less responsive to the Ca ion control [101–103]. The enzymes governing the phosphorylation, specific protein kinases and phosphatases, are in turn themselves regulated by Ca ions and cyclic AMP. Therefore this type of regulation by covalent protein modification is subject to hormonal control as has been shown in the case of heart muscle [104–108]. Reversible phosphorylation of one type of myosin LC also with a molecular weight of 19,000 dalton seems in smooth muscle and non-muscle cells whose contraction response is rather slow when compared with that of striated muscles, to represent a potent control mechanism for the actin-myosin interaction [109–112].

B. Protein Conformational Changes in Myosin

1. Posing the Problem

The postulated movement of the myosin heads which are attached to the actin strands during the contraction cycle, in biochemical terms, requires definite conformational changes to occur in the protein structure. The mechano-chemical trans-

duction process will only be understood when the sequence of conformational changes that occur in the protein can be correlated to the elementary steps in the hydrolytic cycle which ultimately provide the energy. Force generation is thought to be brought about by conformational changes in the cross-bridges in such a way that the myosin heads rotate while they are attached to the actin [63, 113, 114]. The angled attachment to actin as it probably presents the last state in the rotational head movement just before it detaches from actin, has been conclusively established by X-ray diffraction studies both in isolated actomyosin [115] and in intact insect flight muscle [55, 116, 117]. Other states of attachment of the cross-bridges with actin in intact muscle were sought to be induced by binding of the product ADP [118] or ATP analogues to the active centres of the myosin heads [119–122]. The idea for using ATP analogues in order to shift the equilibrium of attached cross-bridges away from the angled position was based on the assumption that these newly observed states of attachment may occur transiently in the cross-bridge cycle. There is however as yet no structural evidence concerning the orientation of attached myosin heads during contraction. Nor does the X-ray diffraction technique allow to differentiate the 2 heads of one myosin molecule which together may form a cross-bridge and which may function together in a concerted way [123, 124].

Some kind of conformational changes would be expected to occur at the far end of the myosin head as a consequence of its physical interaction with actin and during its rotation on the opposite end, at the head's basis where it is attached to the neck region of the myosin rod. Though the active centre is known to be located within the head portion its position is subject to speculation. Nevertheless interaction with actin profoundly affects the enzymic properties of the active centre [125] and on the other hand, it had been suggested that its binding with ATP or one of its analogues may be sufficient to produce the backward stroke of the myosin heads so that the cross-bridge reverts into its rectangular position and is ready again for interaction with the actin filament when so required [120]. If this is true mere binding of a ligand may induce conformational changes in the myosin head region or at its basis. Although the contraction cycle is closely linked to the enzymic cycle by being accompanied by hydrolysis of just one ATP molecule or may be 2 moles of ATP if both heads were involved in the cross-bridge rotation [79], one wonders whether conformational changes associated with ligand binding or ATP hydrolysis can also be observed on isolated myosin. Physico-chemical methods in general have failed to reveal such conformational changes in intact myosin. In part this is certainly due to the large size of the molecule so that possible changes in specific areas would be relatively small and beyond the resolution potential. The existence of 2 distinct conformational states of myosin depending on the presence of either the substrate ATP or the product ADP has, however, been demonstrated by the use of spectral techniques involving ultra violet absorption [126, 127], intrinsic tryptophan fluorescence [128, 129], electron spin resonance [130] and circular dichroism [131]. But the protein is required to be in solution for these studies, i.e. myosin had to be dissolved in at least 0.5 M of salt or alternatively its water soluble subfragments could be examined only. Furthermore high concentrations of nucleotide ligands do interfere with most spectral methods.

Another biochemical approach to gain insight into structural features of proteins

of biological interest employs chemical probing with suitable reagents for specific groups on certain amino acid side chains. The majority of non-polar (hydrophobic) side chains are located in the core of the compact three-dimensional protein matrix [132] and conversely, most polar and electrically charged (hydrophilic) side chains prefer the surface and crevices of the matrix. The protein surface is irregular in contour and inhomogeneous, thus a variety of surface polarities may be expected from non-polar to other regions exceeding the polarity of the aqueous medium itself. The physical and chemical properties of a protein functional group will be strongly influenced by the nature of its local environment. For steric reasons most functional side chain groups are less reactive in proteins than the same groups in isolated amino acids. A smaller number, sometimes no more than one per molecule, may have an even higher reactivity toward certain reagents. Although such a group is often associated with the enzymic activity of a protein, it need not be that particular functional group which is vital for the catalytic role. In part such superreactivity arises from the limitation of conformational populations, i.e. from restricted freedom of rotation. Changes in the microenvironment of such side chain groups may be small in physical terms, stretching a bond by a fraction of an Ångström or twisting it through 20° only. Such changes of chemical groups in a protein could be entirely missed e.g. by present X-ray crystallographic methods, provided the protein concerned can be crystallized at all. This has not yet been achieved with myosin. Further, high energy absorption spectroscopy cannot be expected to yield a definition of the state of groups such as serine-OH or sulfhydryl-SH, and nuclear magnetic resonance methods are not available for their study [133]. Yet such small changes may enhance or reduce the reactivity of a particular group toward a certain reagent considerably.

Since some time it is known that in isolated myosin blockage of thiol groups affects its enzymic properties [134, 135]. It was however not clear how many groups precisely became blocked under different experimental conditions nor was their localization in this large molecule established. Since on the whole, thiol groups are in many instances the most reactive side chain groups in biological macromolecules, monitoring changes in their reactivity has acquired widespread recognition as a useful method for probing protein conformational changes. Distinct changes in reactivity of certain specific thiol groups connected with the enzymic centre have recently been reported for the sodium-potassium transport adenosine triphosphatase of cell membranes [136], aspartate aminotransferase [137], papain [138] and tryptophanase from Escherichia coli [139]. Following the reactivity changes of specific thiol groups in myosin in conjunction with the effect their chemical modification has on the enzymic properties, allowed the characterization of different conformational forms of this enzyme with or without ligand.

This type of conformational characterization has several advantages over any spectral technique. The different conformational forms may be defined independently of a reference state, say the protein without ligand. The method, in particular in its combination with the ensuing enzymic changes as indicator, is extremely sensitive and applicable over a wide range of experimental conditions. It has the potential for characterizing states of myosin at low ionic strength when the protein is in its gel state as it is in muscle, or even when it is combined with actin in more organized systems such as actomyosin or intact myofibrils. Lastly when the specifically reactive thiol groups

have been radioactively labeled it becomes possible to break up the molecule into fragments, isolate the labeled peptides in order to study their structure. Thus it will be possible to localize and understand the chemistry of different functional parts in this fascinating macromolecule involved in movement and mechano-chemical energy transduction.

From a variety of alkylating reagents used, the hydrophilic N-ethylmaleimide (NEM) and iodoacetamide (IAA) proved to be the most suitable for specific reaction with certain thiol groups of myosin. The various methods involved in approaching the problem of characterization of different conformational states of myosin which are of biological interest are described in detail in a number of publications by the author and his coworkers [19, 52, 76, 86, 97, 125, 140–145]. Mainly myosin from fast-contracting rabbit skeletal muscles was used in these studies.

2. Incorporation of Radioactive Alkylation Reagents into Myosin and Isolated Myosin Heads

Alkylation with radioactively labeled reagents was performed on intact myosin and myosin heads which had been separated from the parent molecule by limited proteolytic digestion, under mild conditions with the proteins still being in their native form [76, 143, 145]. When the alkylation reagent was a few times up to a maximum of about a 100 times in excess over the molarity of the protein, it was found that a rather stable

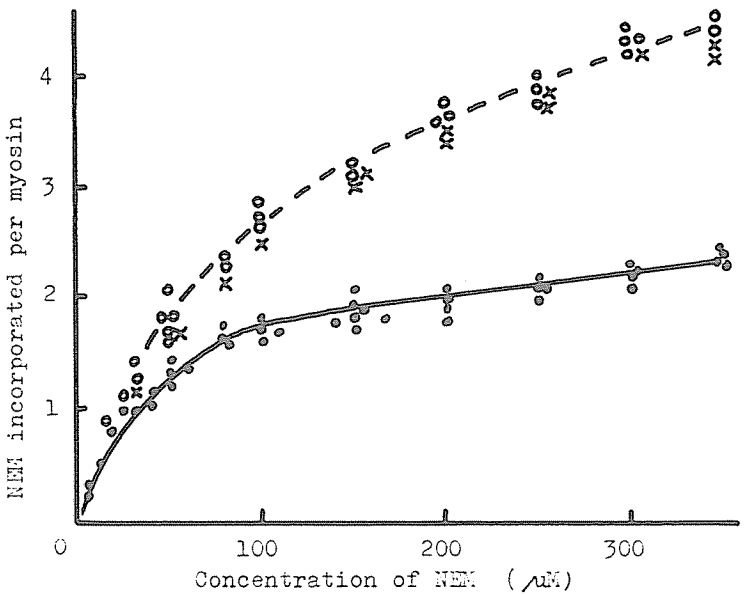


Fig. 9. Incorporation of ^{14}C-NEM into myosin plotted against NEM concentrations. Alkylation was performed on 13.3 μM myosin for 30 min. at pH 7.6 in the presence of 5 mM $MgCl_2$ and 2.5 mM pyrophosphate under different conditions: ● = 0° C and 50 mM KCl; ○ = 25° C and 50 mM KCl; × = 0° C and 0.5 M KCl.

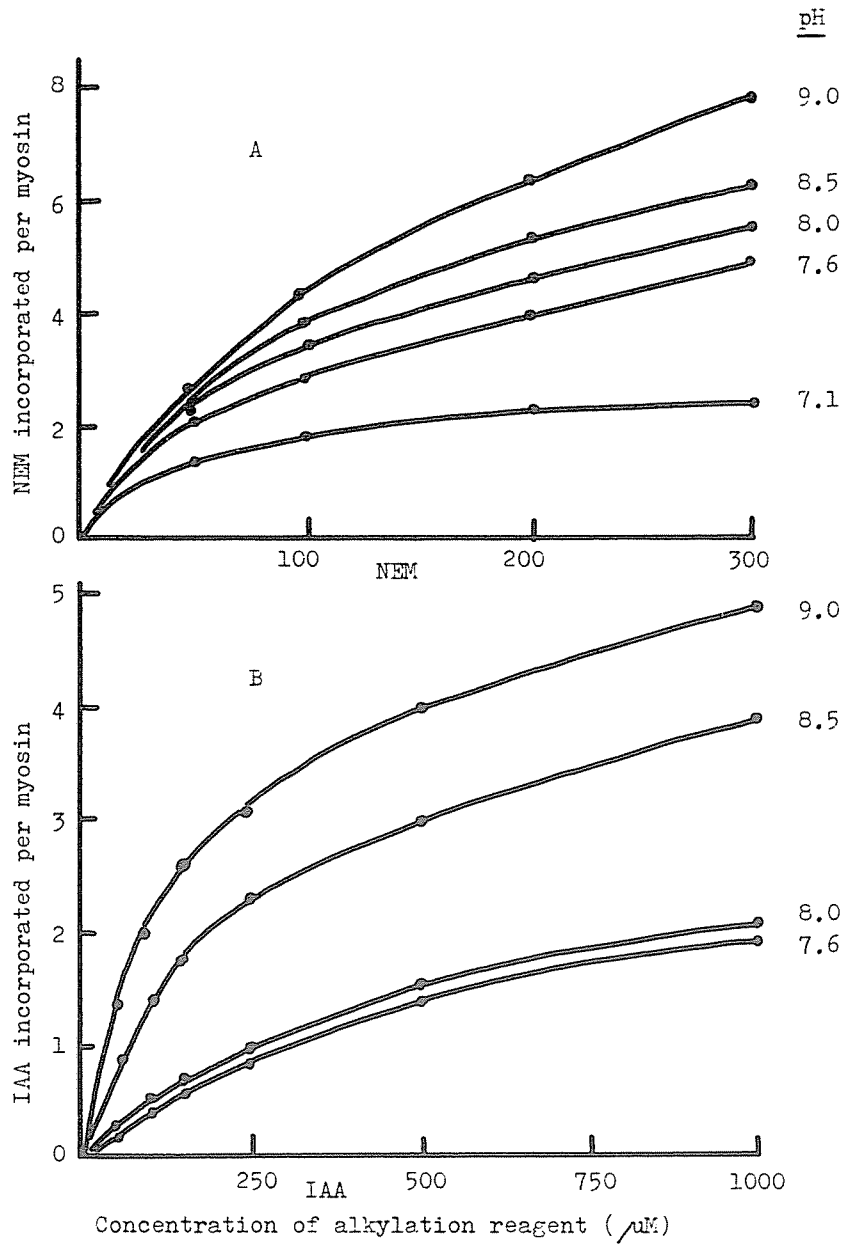


Fig. 10. Incorporation of ^{14}C-NEM (A) and ^{14}C-IAA (B) into myosin plotted against reagent concentrations. Alkylation was performed on 8.4–13.3 μM myosin for 20 min. at 25° C and different pH values in the presence of 10 mM $MgCl_2$ and 5 mM ADP.

level of incorporated radioactivity was established after 5–10 min. The leveling of incorporated radioactivity which develops when plotted against increasing concentrations of the offered alkylation reagent, yields the number of those thiol groups which are most reactive. Considering that intact myosin contains a total of 42 thiol groups [146–148] surprisingly few react readily with NEM or IAA under most conditions. The majority of thiol groups must be located such that they are not available for alkylation unless the protein is denatured.

The precise number of readily reacting thiol groups depends on the conditions during the reaction as well as on the reagent used for alkylation. A level of radioactive incorporation indicating that just 2 thiol groups per myosin are reactive towards NEM was consistently found only when alkylation was carried out at low ionic strength and 0° C independent of whether ligands (pyrophosphate, ADP or ATP) were present or not (fig. 9). As soon as one parameter was changed during the alkylation reaction, e.g. 25° C instead of 0° C or addition of 0.5 M KCl, the degree of radioactive incorporation almost doubled. These results indicate that, all other parameters remaining the same, higher temperature as well as high ionic strength produce a change in the myosin molecule rendering 2 more thiol groups reactive towards NEM. A similar temperature dependent change in the number of readily reacting thiol groups has also been found with myosin from heart muscle [19]. Increasing the pH led to a gradual growing, though still limited, number of thiol groups per myosin which reacted readily with either NEM or IAA in the presence of ADP at 25° C (fig. 10). At all corresponding pH values about 2–3 more molecules of NEM than of IAA were incorporated per myosin, although the concentration of alkylation reagent offered to the protein was

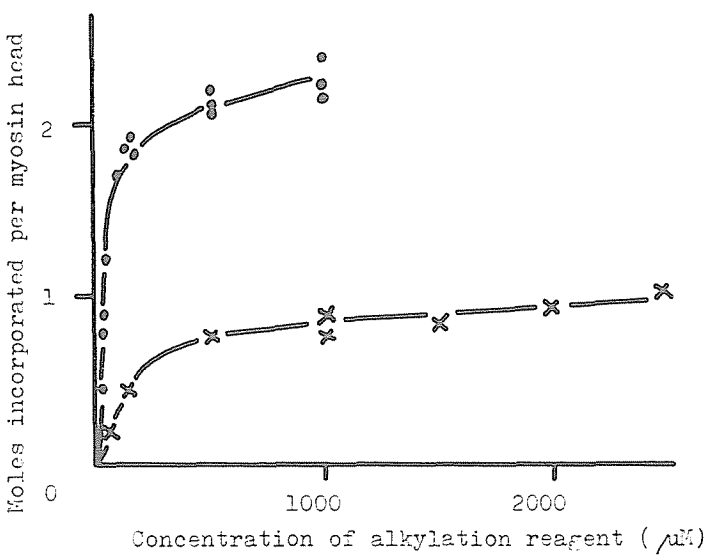


Fig. 11. Incorporation of ^{14}C-NEM and ^{14}C-IAA into isolated myosin heads plotted against reagent concentrations. Alkylation was performed on 3.6–7.5 μM heads at 0° C in the presence of 10 mM $MgCl_2$ and 2.5 mM pyrophosphate with NEM (●) for 20 min. at pH 8.0 and with IAA (×) for 40 min. at pH 8.5.

3 times higher for IAA than in the case of NEM. This effect of pH is to be expected and may occur independently of concurrent protein conformational changes since the nucleophilic thiol groups require their proton-poor form for reaction with alkylation reagents.

In isolated myosin heads again a small number of thiol groups was found to react readily with alkylation reagents, i.e. 2 per head in the case of NEM and one only in the case of IAA (fig. 11). The total number of thiol groups per head has been reported to be about 12 for preparations produced from myosin by proteolytic digestion with trypsin, chymotrypsin or papain [31, 148, 149, 150]. Unlike with myosin the number of readily reacting thiol groups in heads was not increased by higher temperature or high ionic strength. Beside this fundamental difference in the dependence of the readily reacting thiol groups in intact myosin and isolated heads on temperature and ionic strength, there was in both proteins no difference in the number of groups which became blocked whether the alkylation was performed in the presence of the substrate ATP or the product ADP.

3. Functional Differentiation of Blocked Thiol Groups in Myosin and Isolated Myosin Heads

It has been reported earlier that the blockage of thiol groups in myosin has a marked influence on its enzymic properties. Thus it is particularly informative to follow the reactivity changes of thiol groups in conjunction with the ensuing effects on the enzyme function induced by their blockage. Attention has been focused on the Ca-dependent ATPase activity of such modified myosin and 2 classes of reactive thiol groups have been identified in this way. Blockage of the first class (thiol-1 groups) by NEM has an activatory effect, and the subsequent blockage of the second (thiol-2 groups), an inhibitory effect on this activity [151–154]. Blockage of the so-called thiol-2 groups should not affect the Ca-ATPase though it still inhibits the monovalent cation-dependent ATPase activity [155]. It has been recently shown that blockage of thiol-1 groups also leads to activation of the Mg-dependent ATPase but that both types of divalent cation-stimulated ATPase activities (i.e. with Mg or with Ca ions) only display the activatory effect when the enzyme-tests are performed at high ionic strength and at a temperature above 20° C [143]. However, blockage of either of these 2 classes of thiol groups invariably inhibits the K-dependent ATPase when tested in the presence of EDTA in order to remove trace amounts of divalent cations. Therefore they will both be referred to as essential thiol-1 and essential thiol-2 groups. We described a third class (thiol-3 groups) comprising a number of thiol groups whose blockage does not affect the enzymic properties of myosin and which are rendered reactive towards NEM under specified conditions only [76, 143]. Depending upon the conditions during the alkylation reaction in most cases a limited number of different types of thiol groups become modified. Following the progressive degree of blockage in conjunction with subsequent analysis of the mono- and divalent cation-stimulated ATPase activities, it was possible to define the sequence of reaction of the different types of groups and thus to characterize in this way a number of conformational states in the native protein.

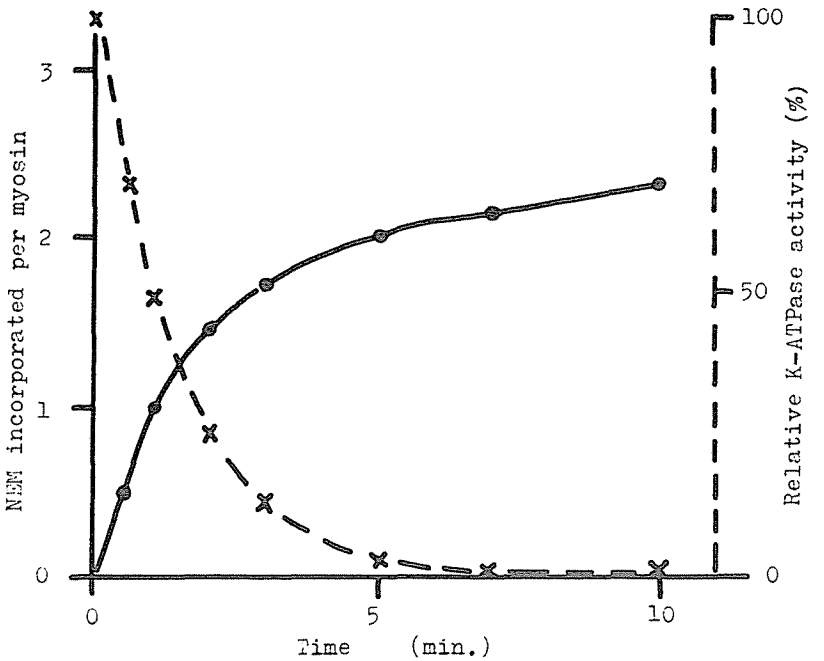


Fig. 12. Time-course of incorporation of ^{14}C-NEM into myosin and concomitant inhibition of K-ATPase. Prior to the ATPase tests alkylation with 50 μM NEM was performed on 5.2 μM myosin in the presence of 5 mM $MgCl_2$ and 5 mM ADP at 0° C and pH 7.6 for different periods of time. ● = NEM incorporated per myosin; × = K-ATPase activity.

A time-course experiment shows that alkylation in the presence of Mg-ADP at 0° C and low ionic strength with a 10-fold molar excess of NEM led within 5 min. to blockage of 2 thiol groups per myosin. Concomitantly as the degree of thiol group blockage proceeds, the K-dependent ATPase activity decreases becoming fully abolished just when 2 NEM molecules were incorporated per myosin (fig. 12). The same result was obtained when alkylation was performed in the presence of Mg-pyrophosphate indicating that in both cases 2 essential thiol groups were the most reactive. Since each myosin head bears an active centre, an essential thiol group in each head is expected to be blocked when the K-dependent ATPase was destroyed. Isolated heads obtained from such modified myosins by digestion with chymotrypsin, indeed had just one molecule of NEM incorporated per mole of heads.

In order to decide which class of essential thiol groups (i.e. thiol-1 or thiol-2 groups) became essentially blocked, the Ca-dependent ATPase had to be considered too. Plotting the enzymic activities as a function of degree of thiol group blockage allows the readily reacting groups to be classified on the above mentioned criteria. Inspection of fig. 13 reveals immediately that the first group which reacts with NEM is of the same class in both cases with pyrophosphate or with ADP. The K-ATPase is reduced to 50% and the Ca-ATPase increased to the same extent when one thiol group has been

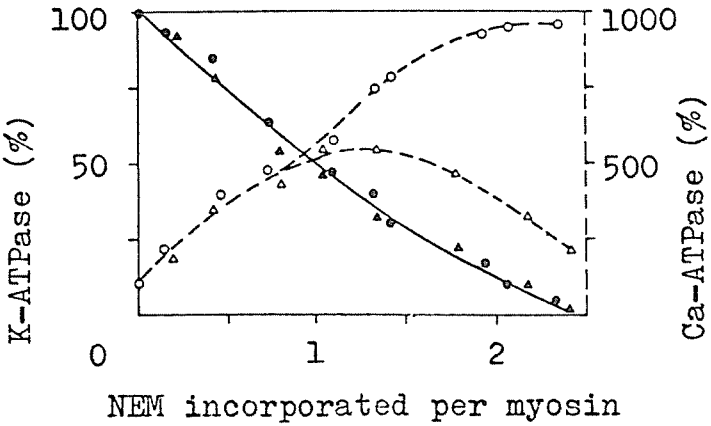


Fig. 13. Effect of incorporation of ^{14}C-NEM into myosin on relative ATPase activities. Prior to the ATPase tests alkylation was performed for 15 min. at 0° C and pH 7.6 in the presence of 5 mM $MgCl_2$ and 2.5 mM pyrophosphate (●, ○) or ADP (▲, △). Full symbols = K-ATPase; open symbols = Ca-ATPase.

blocked, hence in both cases it was a thiol-1 group that reacted first. Equally well can be seen that the second group to become blocked was a different one in the 2 cases. While the K-ATPase was fully abolished in both cases when the second thiol group was blocked, the Ca-ATPase further increased to the double only in the case where the alkylation reaction had been carried out in the presence of the ligand pyrophosphate. So in this case 2 thiol-1 groups must have been blocked successively, i.e. one on the first head followed by the second one of the other head. In the presence of ADP the second group to become blocked must also reside in the second head because of the full inactivation of the K-ATPase caused by its blockage. In contrast however, in this case the Ca-ATPase is not further activated but remains almost unchanged or reverts somewhat. Hence this second readily reacting thiol group fits the definition of the so-called essential thiol-2 groups whose blockage does not activate the Ca-ATPase but still abolishes the K-ATPase.

If the alkylation with NEM was carried out at 25° C where 4 thiol groups react readily, the pattern for the first 2 groups to react as described at 0° C, was preserved (fig. 14). In the case of pyrophosphate the maximum Ca-ATPase activity was reached when the K-ATPase was fully inactivated and that happened when 2 thiol-1 groups per myosin had reacted with NEM. The further 2 groups which became blocked had hardly any effect on the high Ca-ATPase activity. So the additionally reacting groups belong to the so-called non-essential thiol-3 class. With the ligand ADP, however, after a thiol-1 group has been blocked on one myosin head, again an essential thiol-2 group must have reacted next on the other head to account for the facts that the K-ATPase was further inactivated and the Ca-ATPase remained at only half its maximal potential activity. The third thiol group blocked seems to be a thiol-2 on the first head since the Ca-ATPase activity reverted from its intermediate level (induced by blockage of the first reacting thiol-1 group on head one) to about its starting activity or below.

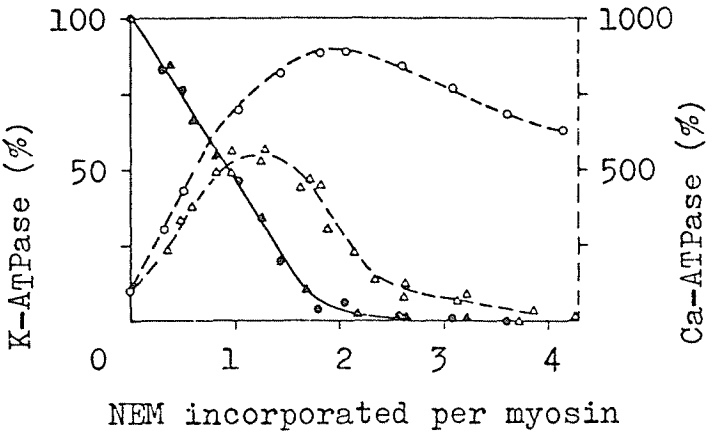


Fig. 14. Effect of incorporation of ^{14}C-NEM into myosin on relative ATPase activities. Prior to the ATPase tests alkylation was performed for 20 min. at 25° C and pH 7.6 in the presence of 5 mM $MgCl_2$ and 2.5 mM pyrophosphate (●, ○) or ADP (▲, △). Full symbols = K-ATPase; open symbols = Ca-ATPase.

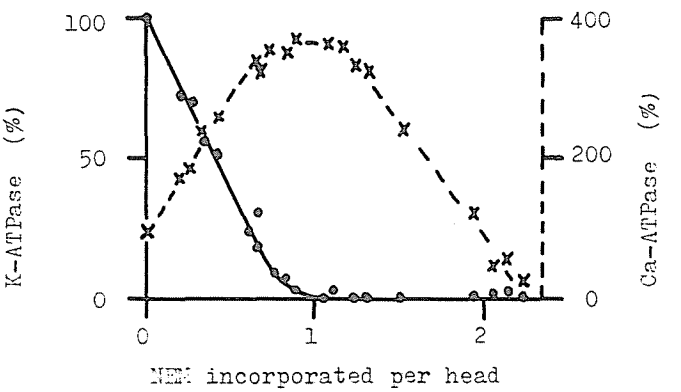


Fig. 15. Effect of incorporation of ^{14}C-NEM into isolated myosin heads on relative ATPase activities. Prior to ATPase tests alkylation was performed on 7.5 μM heads for 15 min. at 0° C and pH 8.0 in the presence of 10 mM $MgCl_2$ and 2.5 mM pyrophosphate. ● = K-ATPase; × = Ca-ATPase.

The fourth that becomes blocked successively must then be another essential thiol group of head number 2 since by now both monovalent and divalent cation-dependent ATPase activities are fully destroyed. Although the principal order of reaction for the 4 readily reacting thiol groups can be deduced in this way, the results also indicate that their sequence of blockage does slightly overlap.

A couple of important implications can be drawn from the above results. First there are 2 essential thiol-1 and probably 2 essential thiol-2 groups per myosin. Since a myosin molecule contains 2 heads each with an active site there would just be one thiol-1 and one thiol-2 group associated per active centre. That isolated myosin heads still contain essential thiol-1 and thiol-2 groups was indicated by the biphasic response

of the Ca-ATPase activity which first increased and subsequently decreased with time of alkylation with non-radioactive NEM [156]. By using radioactively labeled NEM for alkylation in the presence of pyrophosphate we showed that blockage of one thiol-1 group followed by modification of one thiol-2 group per head accounted for both the rising and the falling part of the biphasic Ca-ATPase curve (fig. 15). It has to be noted that in isolated heads alkylation with NEM in the presence of pyrophosphate did not lead to blockage of non-essential thiol-3 groups as it was the case in intact myosin.

The second implication portends that in intact myosin binding of the nucleotide ADP leads to some type of interaction between the 2 myosin heads rendering the thiol-2 of head number 2 more reactive than its thiol-1 group provided thiol-1 of head one is already blocked by NEM. The nucleotide moiety seems to be required for this information that has to pass from one head to the other since it was also observed when alkylation was performed in the presence of ATP [143], not however with the ligand pyrophosphate.

Kinetic analysis of the 2 readily reacting thiol groups and concomitant inactivation of the K-ATPase at 0° C and in the presence of ADP confirmed the above conclusion

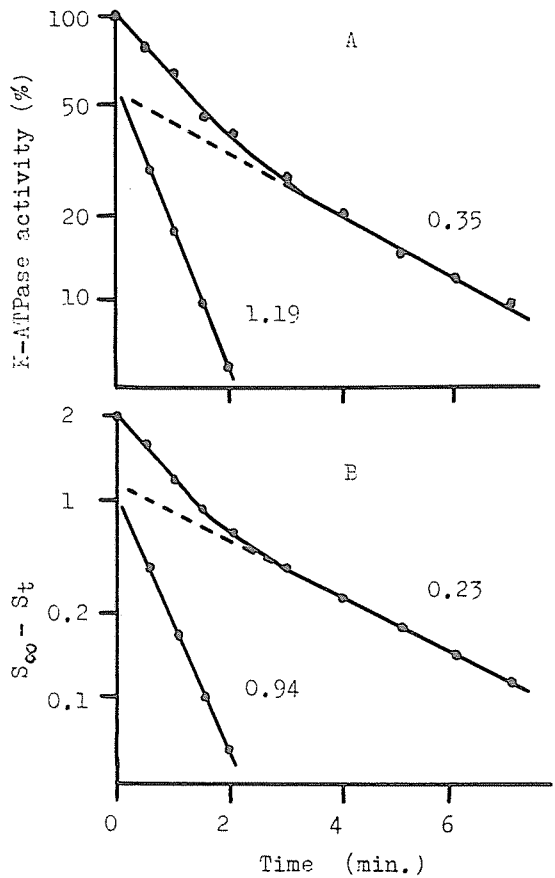


Fig. 16. Logarithmic plot of loss of K-ATPase activity (A) and loss of readily reacting thiol groups (B) versus time. Prior to ATPase tests alkylation was performed with 54 μM ^{14}C-NEM on 7.6 μM myosin at 0° C and pH 7.6 in the presence of 7.5 mM $MgCl_2$ and 5 mM ADP. Values given on lines are rate constants (min^{-1}). Further explanations are given in the text.

that 2 different types of essential groups reacted with NEM under these conditions. When the data were plotted semilogarithmically according to KOSHLAND et al. (1958) [157], both reactions could be resolved into 2 processes with different rate constants (fig. 16). The lines for the fast process were obtained subtracting the extrapolated lines for the slow process assuming both represented pseudo first order reaction. $S_\infty - S_t$ on the ordinate indicates the difference between the maximum number of thiol groups per myosin blocked by NEM at 0° C ($S_\infty = 2$) and at time t (S_t). The 2 phases of inactivation of the K-ATPase paralleled closely the first fast reacting thiol-1 and the second slower reacting thiol-2 group. In contrast in the presence of pyrophosphate when the thiol-1 groups on both myosin heads become blocked only one reaction rate constant was found for the alkylation and ATPase inactivation (fig. 17). Average rate constants from 3 experiments each are summarized in table 3.

It had been reported previously that the presence of a nucleotide facilitated alkylation of thiol-2 groups in myosin [155, 158, 159] without, however, appreciating the precise sequence of events involving cooperativity between the 2 myosin heads as described here.

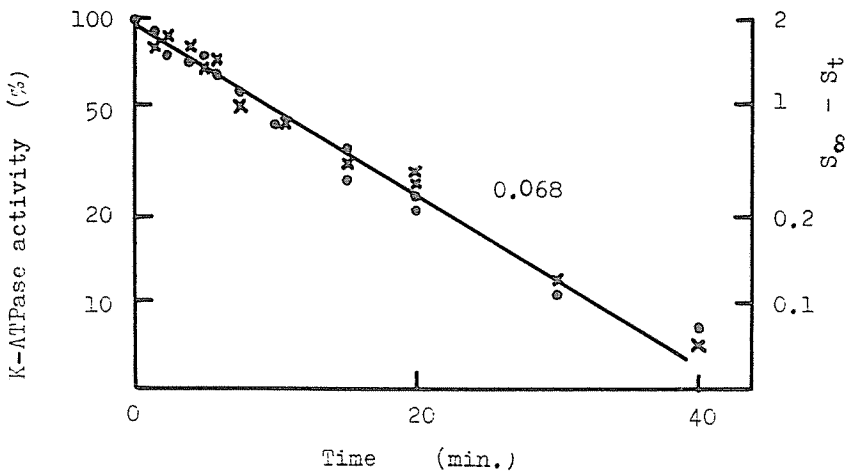


Fig. 17. Logarithmic plot of loss of K-ATPase activity (●) and loss of readily reacting thiol groups (×) versus time. Prior to ATPase tests alkylation was performed as described in fig. 16 but in the presence of 2.5 mM pyrophosphate instead of ADP. Rate constant for both reactions equals 0.068 min^{-1}. Further explanations are given in the text.

Table 3. First order rate constants of K-ATPase inactivation and alkylation of the 2 readily reacting essential thiol groups of myosin by ^{14}C-NEM at 0° C and low ionic strength

Ligand added during alkylation reaction		Rate constant (min^{-1}) k_1	k_2
Mg-ADP	Thiol reactivity	1.20	0.20
	K-ATPase	1.24	0.34
Mg-pyrophosphate	Thiol reactivity	0.055	–
	K-ATPase	0.056	–

4. Myosin Conformational Changes Induced by ATP Hydrolysis

As outlined in the previous section it was possible to describe several different conformations myosin adopted depending on the ligand and experimental conditions in terms of number and classes of readily reacting thiol groups based on incorporation of radioactively labeled NEM and enzymic properties of the modified molecule. Some of these results are summarized in table 4. For higher degrees of incorporation than 4 NEM per myosin at 25° C a 100–500 times molar excess of the reagent over protein had to be used. Under the mild alkylation conditions employed at 0° C and low ionic strength never more than around 2 NEM molecules were incorporated per myosin. But at 25° C at low and at high ionic strength a larger number of thiol groups was readily blocked by NEM and under most conditions among the first 4 reacting thiols was a variable amount of non-essential thiol-3 groups. Most of those myosin conformations described in table 4 which were obtained at 25° C and low ionic strength are of biological interest insofar as they represent 4 of the 7 intermediary states occurring during hydrolysis of ATP by isolated heads. The long lived species within this hydrolytic cycle is known to be an enzyme-product complex, M**ADPP$_i$ [81, 160] and it is this molecular state which prevails when alkylation is performed in the presence of ATP. It can well be distinguished from the complex originating from direct binding of the product ADP to myosin, M*ADP. Although it has recently been established that the species M*ADPP$_i$ exists in series between M**ADPP$_i$ and M*ADP in the hydrolytic scheme, it cannot be observed under steady-state conditions as it is too short lived [75].

$$\mathrm{M}+\mathrm{ATP} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{MATP} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \mathrm{M^*ATP} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{M^{**}ADPP_i} \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} \mathrm{M^*ADPP_i}$$

$$\underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} \mathrm{M^*ADP}+\mathrm{P_i} \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} \mathrm{MADP} \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} \mathrm{M}+\mathrm{ADP}.$$

The product complex M*ADP may however be examined as it is identical to that formed by binding of ADP to the enzyme. A similar problem poses the enzyme-substrate complex M*ATP.

It would be desirable to obtain information about the conformation of this complex since it has been shown that the substrate-loaded myosin heads have a low affinity to actin and dissociate rapidly before ATP is split [79]. But in turn the ATP hydrolysis step is also fast with a rate constant of 100–200 sec^{-1}. Thus in order to be able to observe this enzyme-substrate complex ATP analogues had to be employed which shifted the rate limiting step in the hydrolytic cycle. To this end we used 5′-adenylylimidodiphosphate (APPNP) which is bound but not cleaved at the active centre [161] and adenosine-5′-0-(3-thiotriphosphate) (ATPγS) which although it is split by myosin, also causes the substrate-complex to be the predominant species [162]. The results for APPNP in table 4 show that the enzymesubstrate complex differs in its conformation from both types of product complexes (i.e. that originating from ATP hydrolysis = M**ADPP$_i$ and that formed on simple binding of ADP = M*ADP). ATPγS yielded the same results as APPNP.

The characteristic patterns of the mono- and divalent cation-dependent ATPases

resulting from progressive thiol group blockage of myosin at 25° C and low as well as high ionic strength in the presence of either ADP or ATP led to the clear distinction between the long lived product complex when ATP is turned over, and the ADP binding complex (table 4). The most obvious difference is that in the case of ATP (i.e. $M^{**}ADPP_i$ being the predominant species) the first reacting thiol group does not affect the enzymic properties, hence it belongs to the non-essential thiol-3 class. Therefore the typical inactivatory and activatory effects with progressive thiol group blockage is delayed until more groups become modified than in the case of ADP (= M*ADP). With ATP in fact up to 3 non-essential thiol-3 groups are among the first 4 which react with NEM while with ADP the essential thiol groups are the most reactive ones. This general finding that a number of thiol-3 groups are rendered more reactive than the essential ones when ATP is turned over, also holds for myosin which is dissolved in high salt concentration. Under these latter conditions with ADP there is only one thiol-3 group among the first 4 reacting groups.

It had to be explained why at 0° C no difference was found between the 2 cases. We could show that the difference at 25° C gradually diminished as the temperature during alkylation with either ADP or ATP was lowered below 15° C and disappeared completely at around 5° C [76]. Furthermore the pH dependence of the divalent cation-dependent ATPases of myosin was also found to change gradually by lowering the temperature so that the relative activity minimum between pH7 and 8 at 25° C was transformed in an activity maximum below 10° C. A third parameter, the activation energy for the overall hydrolytic process of the divalent cation-dependent ATPases of myosin again changed from lower to higher values in going from 15° C down to 0° C [76]. Such changes in pH dependence and activation energy with temperature are often indicative of a change in the enzyme mechanism, i.e. a shift of the rate limiting step in the hydrolytic scheme [163]. With such a change in the rate limiting step the predominant enzyme species in the steady-state would naturally also be shifted to another one which eventually would be recognized as a different conformation. This is exactly what we observed by following the alkylation reaction in the presence of ATP when it is turned over by the myosin at different temperatures. From the fact that the patterns of thiol group blockage at 0° C with ADP or ATP are identical it can be further deduced that the rate limiting step must have moved from the isomerization step of $M^{**}ADPP_i \rightleftharpoons M^{*}ADPP_i$, being rate limiting at 25° C, to a step further to the right in the hydrolytic scheme so that e.g. M*ADP may now become the predominant species. Kinetic studies having shown that indeed M*ADP seems to be the prevailing intermediate during hydrolysis of Mg-ATP at low temperature [57, 164, 165] confirm our interpretation.

Most studies referred to so far concerned myosin from fast-contracting rabbit skeletal muscles. Less is known about the myosins from slow-contracting skeletal muscles and from heart. Both slow and cardiac myosin do differ from fast myosin in a number of properties. They have 2 types of LC only, their ATPase activities are about one third of that from fast myosin [166] and in particular their HC are chemically different from those of fast myosin. Both types of slow myosins do not contain 3-methylhistidine in their HC [167–169], exhibit a different electrophoretic mobility [170] and give rise to different peptide patterns upon tryptic digestion [171] or chemical

Table 4. Integral numbers of thiol groups blocked per myosin during alkylation in the presence of $MgCl_2$ at pH 7.6 with different ligands in conjunction with ATPase characteristics of such blocked myosins

	Ligand	Total thiols blocked	Total thiols blocked when K-ATPase inact.	Ca-ATPase at max.	Ca-ATPase inact.	Relative Ca-ATPase at max.	Reaction sequence of thiols of different classes First	Second	Third	Fourth	Thiol-1 and thiol-2 blocked	Thiol-3 blocked
A	None	2	2	2	–	100	1	1	–	–	2	–
	PP*	2	2	2	–	100	1	1	–	–	2	–
	ADP	2	2	1	–	55	1	2	–	–	2	–
	ATP	2	2	1	–	50	1	2	–	–	2	–
	APPNP*	2	2	2	–	80	1	1	–	–	2	–
B	None	4	4	4	–	100	3	1	3	1	2	2
	PP	4	2	2	–	100	1	1	3	3	2	2
	ADP	4	2	1	4	45	1	2	2	1	4	–
	ATP	8	6	3–4	8	45	3	1	3	3	4	4
	APPN	4	2	2	4	85	1	1	2	2	4	–
C	None	4	4	4	–	100	3	1	3	1	2	2
	PP	4	4	4	–	90	3	1	3	1	2	2
	ADP	8	6	1–2	8	30	3	1	2	1	4	4
	ATP	12	10–11	4–5	12	30	3	3	1	3	4	8
	APPNP	10	8	4	10	55	3	1	3	3	4	6

A = 0° C and 30 mM KCl
B = 25° C and 30 mM KCl
C = 25° C and 0.6 M KCl

* PP = Pyrophosphate
APPNP = 5′-Adenylylimidodiphosphate

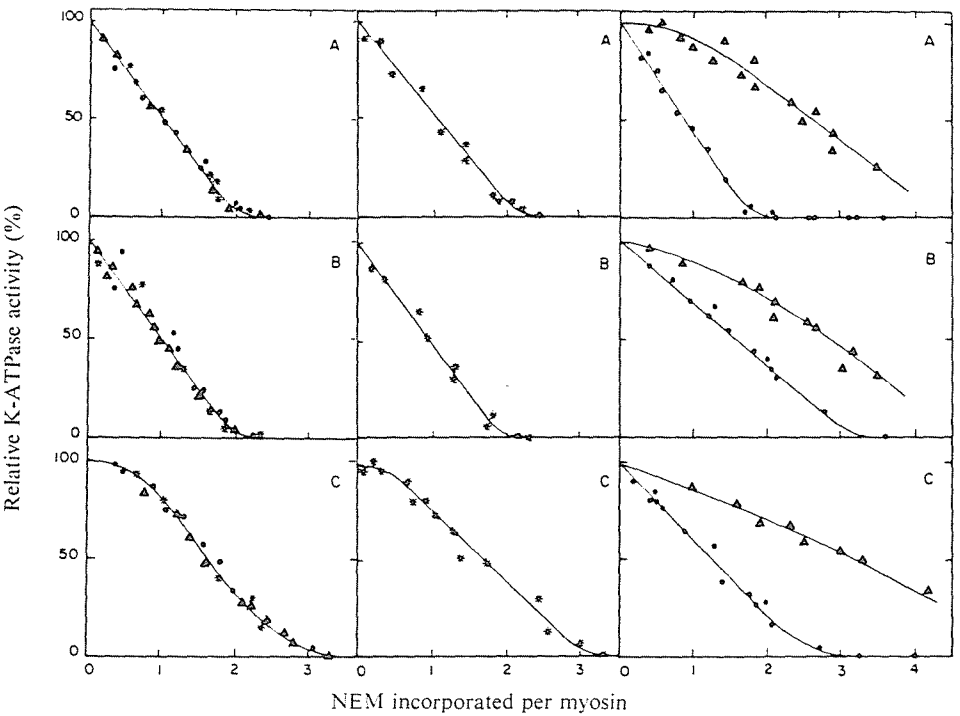


Fig. 18. Effect of thiol group blockage by ^{14}C-NEM at 0° C and 25° C on the K-ATPase activity of myosins from fast, slow and cardiac muscles. Prior to ATPase tests alkylation was carried out on 5–15 μM myosin for 15 min. at pH 7.6 and low ionic strength in the presence of 5 mM $MgCl_2$ and 2.5 mM pyrophosphate (*), 5 mM ADP (●) or 5 mM ATP (▲). Left column = alkylation at 0° C; middle and right columns = alkylation at 25° C. A = fast myosin; B = slow myosin; C = heart myosin.

cleavage [172]. While myosin from slow-contracting muscles is reported to contain also 41–42 thiol groups per molecule [173], this number varies for cardiac myosin somewhat in the literature, averaging at around 37 per mole [148, 174–176]. All these findings indicate that both HC as well as LC differ in their primary structure between the fast and the slow types of myosins and the question therefore arises whether similar conformational changes can be observed in the slow myosins as in the fast one associated with the hydrolytic cycle at 25° C.

Fig. 18 shows the effect of progressive thiol group blockage with NEM on the K-ATPase of different types of myosins. The alkylation reaction was performed in all cases at low ionic strength at 0° C and at 25° C in the presence of either pyrophosphate, ADP or ATP. The precise number of thiol groups which were needed to be blocked in order to abolish the K-ATPase was in the 2 slow myosins depending on the ligand and temperature the same as in fast myosin or differed only by one. It had been argued elsewhere that both slow myosins may contain 2 essential thiol-1 and 2 essential thiol-2 groups as does fast myosin [19]. That the number of groups to be blocked for

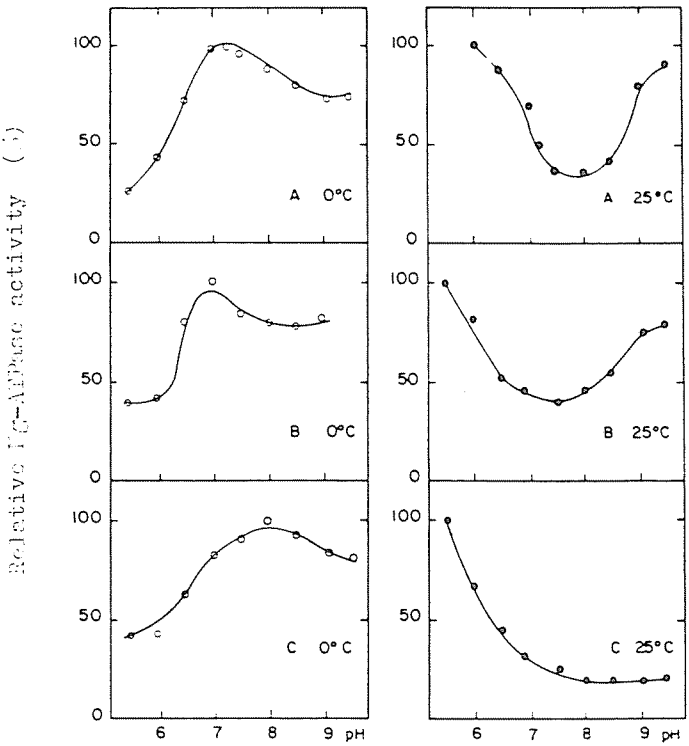


Fig. 19. pH dependence of the Mg-ATPase at 0° C and 25° C of myosins from fast (A), slow (B) and cardiac (C) muscles. Mg-ATPase tests were carried out in presence of 5 mM $MgCl_2$ and 2.5 mM ATP and 50 mM buffer (Tris-maleate for pH 5.5–7.0 and Tris-Cl for pH 7.6–9.5).

inactivation of the K-ATPase nevertheless differed significantly in some cases supports the findings cited from the literature above which indicate structural heterogeneity between fast and slow myosins. Despite of these differences, hydrolysis of ATP induced similar conformational changes at 25° C in the slow myosins as in the fast one. In both slow myosins in the presence of ATP, as with fast myosin, around 5–6 thiol groups had to be modified before the K-ATPase was fully inactivated indicating that in all cases non-essential thiol-3 groups became more reactive as a consequence of the hydrolysis. And again this difference in conformation when compared to that one in the presence of ADP, gradually disappeared on lowering the temperature down to 0° C (fig. 18). Also the pH dependence of the slow myosins was in a similar way affected by temperature like with fast myosin (fig. 19).

Taken together these findings indicate that also in slow myosins the same isomerization step in the hydrolytic cycle is rate limiting and gives rise to a steady-state conformation in which non-essential thiol-3 groups are reactive and that on lowering the temperature to 0° C this rate limiting step is changed in the same way as in fast myosin.

5. Subunit Distribution and Localization of Specific Thiol Groups in Myosin

For localization of the thiol groups which have been identified by the effect of their blockage by either NEM or IAA on the enzymic properties of both myosin and isolated heads, electrophoresis on polyacrylamide gels in a dissociating medium containing sodium dodecyl sulfate was used. In the majority of these studies NEM rather than IAA was employed because the number and types of thiol groups that may be blocked by IAA is limited and its reaction with myosin always involves mixtures of thiol-1 and thiol-3 groups. In particular the thiol-2 groups did not react with IAA under any conditions neither in intact myosin nor in isolated heads. In heads only the thiol-1 group reacted with IAA though a 500 molar excess of the reagent was offered to the protein at pH values up to 9.0. The degree of incorporation did not exceed 1.3 moles of IAA per head even when 1 M urea was present in the reaction mixture. That the thiol-2 group was still intact in heads labeled with IAA, could be demonstrated by carrying out a subsequent alkylation step with NEM. Additional incorporation of around one mole of NEM led to inactivation of the Ca-ATPase that was at its activated level since the thiol-1 group had previously been blocked by IAA.

Myosin heads could be prepared in different ways by digestion with trypsin from myofibrils [177] and by digestion of myosin with papain [36] or chymotrypsin [178]. With all preparations the ATPase pattern typical for subsequent blockage of thiol-1 followed by thiol-2 was obtained on alkylation with NEM. But never in heads any appreciable amount of thiol-3 groups reacted with NEM although the differently prepared heads are reported to contain a total of around 12 thiol groups [31, 148, 149, 150]. It was therefore of interest to discover whether the thiol-3 groups which under certain conditions are the most reactive ones in intact myosin are present in the isolated heads or not.

After alkylation of intact myosin with radioactively labeled NEM under appropriate conditions the degree of incorporation and ATPase activities were determined. Then the myosins were subjected to electrophoresis in sodium dodecyl sulfate (SDS) and after staining the subunit protein band pattern recorded. For assessment of radioactivity incorporated into the electrophoretically resolved subunits, the polyacrylamide gels were sliced into 2 mm sections. From the remainder of the modified myosin samples heads were prepared by digestion with chymotrypsin and in the isolated heads again the degree of radioactive incorporation was estimated, and the protein resolved electrophoretically into its subunits. The recovery of radioactivity from gels of both myosin and isolated heads which were sliced and counted after fixation and staining was between 70 and 95%. In gels which were sliced and counted immediately after the electrophoretic run without prior fixation or staining, the recovery was on average 99%.

Fig. 20 (A–D) shows densitometric tracings of myosin and head preparations. The crude head pattern showed invariably complete loss of LC-2 but an additional band arising from a degraded fragment migrating faster than LC-3. The band pattern in SDS electrophoresis from head preparations of differently modified parent myosins looked identical, the relative areas of the stained protein bands remained constant and

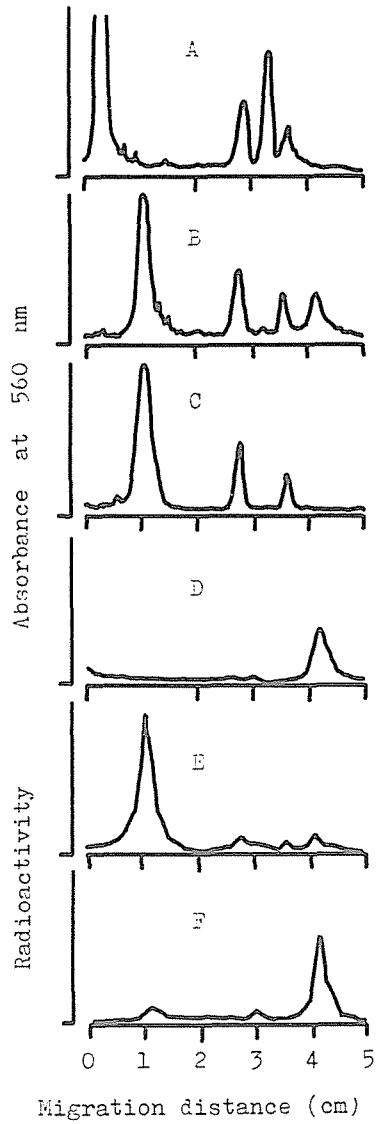


Fig. 20. Electrophoretograms of myosin and isolated head preparations (A–D) and distribution of radioactivity over subunits of heads prepared from myosins which had previously been alkylated with ^{14}C-NEM in the presence of either Mg-ADP or Mg-ATP. SDS electrophoresis was performed on 7.5% polyacrylamide gels. (A) = myosin (HC, LC-1, LC-2 and LC-3); (B) = crude heads (head HC, LC-1, LC-3 and 11,000-fragment); (C) = purified heads (head HC, LC-1 and LC-3); (D) = isolated 11,000-fragment. (E–F) radioactivity recovered from gels of crude heads prepared from myosin containing 1.60 moles ^{14}C-NEM incorporated in the presence of Mg-ADP = (E) and from myosin containing 1.19 moles ^{14}C-NEM incorporated in the presence of Mg-ATP = (F).

on mixing the preparations their protein bands comigrated. From comparison of migration distances of the proteins from myosin and heads with those of proteins with known molecular weight the following apparent molecular weights were calculated: myosin HC = 200,000, head HC = 90,000, LC-1 = 24,800, LC-2 = 17,500, LC-3 = 14,700 and the degraded fragment = 10,800 dalton. It has to be mentioned here that the apparent molecular weights of the light chains based on electrophoretic mobility do somewhat deviate from the chemical molecular weights as determined from the

amino acid sequence [179, 180]. The degraded 11,000 dalton fragment could be separated from the heads by ion exchange chromatography on DEAE-Sephadex and its removal did not affect the enzymic properties nor the alkylation results of the heads indicating that it is not an integral part of them. Estimates of the molar ratio of the 11,000 dalton fragment in crude head preparations on the basis of staining intensity of the electrophoretic bands indicated that there is just about one such fragment per isolated head. Since the LC-2 was lost during preparation of the heads the question arose whether the 11,000-fragment represented a degradation product of this type of myosin light chain.

The major portion of radioactivity incorporated in the presence of pyrophosphate or a nucleotide was recovered consistently in the heavy chain band of myosin. On average around 10% and often less radioactivity was found in all 3 types of LC together. Therefore the distribution of radioactivity over the HC and the 11,000-fragment in the isolated heads was of particular interest. It became obvious that when myosin was labeled with NEM under several experimental conditions at 25° C, the more thiol-3 groups had been blocked the more radioactivity was recovered in the 11,000-fragment. In certain experiments the 11,000-fragment contained 20–25 times more radioactivity than an equimolar amount of the LC-2 in the parent myosin molecule. This already lets suggest that the 11,000-fragment originated from the myosin HC and not from the degraded LC-2. In addition there was a marked difference in the recovery in both the head HC and 11,000-fragment between those cases where up to 2 moles of NEM were incorporated per myosin in the presence of Mg-ADP or Mg-ATP at 25° C and low ionic strength. 97% and 86% of the total radioactivity was incorporated into the HC of the parent myosins in the presence of ADP and ATP, respectively.

After digestion with chymotrypsin, however, a reciprocal distribution of the radioactivity over the head HC and the 11,000-fragment was observed (fig. 20, E and F). While the majority of counts remained on the head HC in the case of Mg-ADP, the amount recovered on the 11,000-fragment was 3–4 times larger than that on the HC in the case of Mg-ATP. This result confirms the suggestion that the 11,000-fragment must be derived from the myosin HC. Moreover, it can be concluded that during hydrolysis of Mg-ATP at 25° C a different part of the myosin HC, containing the 11,000-fragment with its thiol-3 groups, becomes heavily labeled than in the presence of the product Mg-ADP. Since in this latter case only thiol-1 and thiol-2 groups were blocked in the parent myosin the 90,000 dalton head HC must contain both types of essential thiol groups. Finally the reported results allow the conclusion that the non-essential thiol-3 groups residing in the 11,000 dalton fragment are not integrated in the isolated functioning myosin heads.

A molecular weight of around 120,000 dalton for heads prepared in the same way by chymotrypsin has been found in ultracentrifuge determinations [178]. Since isolated heads were found to contain just one LC-1 or alternatively one LC-3 per head, after subtraction of the LC a molecular weight of about 100,000 dalton would be expected. On electrophoresis of such heads, however, only one sharp protein band was observed with a molecular weight around 90,000 dalton. It can therefore be concluded that the 11,000-fragment must originate from one end rather than from somewhere in the

middle of the head HC. In the latter case one would expect a number of bands with molecular weights considerably smaller than 90,000 dalton.

It is known that the N-terminal amino groups of both myosin HC are in the head region and that they are acetylated [181]. In addition, the N-terminal amino groups of LC-2 [85] as well as those of LC-1 and LC-3 [180], have also been shown to be blocked. Head HC, LC-1 and LC-3 and the 11,000-fragment from head preparations were separated from each other and purified by ion exchange chromatography on DEAE-Sephadex and gel permeation in 6 M guanidine-HCl on Sephadex G-100 [141] and separately subjected to the Edman degradation procedure. Free N-terminal amino acids could only be liberated from the purified 11,000 dalton fragment and were identified by thin layer chromatography and amino acid analyses as Asp, Ala, and in lesser amounts Leu and Lys. Identical results were obtained from 3 different preparations of the 11,000 dalton fragment. On the other hand, the fact that no free N-terminal amino acids could be liberated from the head HC indicates that their acetylated N-terminal ends have been preserved during the chymotryptic digestion. This is in agreement with WEEDS and his coworkers (1977) [182] who isolated the N-terminal cyanogen bromide peptides from the HC of such head preparations. Since only the 11,000-fragment contains unblocked N-terminal amino groups, it must have been cleaved off from somewhere close or more distant on the C-terminal side of the 90,000 dalton head part of the myosin HC.

The amino acid composition has so far only been reported for isolated heads including a somewhat variable degree of the different types of LC but probably mainly LC-1 and LC-3. By subtracting the amino acids contributed by these LC the likely amino acid composition of the head HC was calculated averaging the determinations of different authors [31, 146, 148–150]. When compared with our direct determination of the amino acid composition on 5 preparations of the 90,000 dalton head HC the agreement is rather good (table 5). A cysteine content of 10 per mole was found among which should be the essential thiol-1 and thiol-2 groups. A cyanogen bromide peptide with an apparent molecular weight of around 10,000 dalton containing the radioactively labeled essential thiol groups could indeed be isolated from the 90,000 dalton head HC and purified by successive gel permeation and ion exchange chromatography. It contains just the 2 essential thiol groups and its amino acid composition corresponds to that of a cyanogen bromide peptide which had been prepared from intact myosin HC and whose amino acid sequence has recently been established by ELZINGA (1977) [183]. Amino acid analysis from 4 preparations of the 11,000-fragment revealed that it contains 2 cysteines per mole including the non-essential thiol-3 groups. The amino acid composition of the entire myosin rod [31] comprising the light meromyosin and heavy meromyosin subfragment-2 part (but devoid of the 2 heads) is also given in table 5. It can be seen immediately that the alpha-helical rod part of myosin does not contain any proline, whereas the 11,000-fragment contains 5 proline residues per mole. Beside the isolated light meromyosin tail which is lacking proline [184–186], in particular subfragment-2 representing the neck region of the myosin molecule, has also been reported to contain no proline residues at all [31, 148, 187]. Both these myosin fragments represent completely alpha-helical polypetides [186, 188–190]. This makes it impossible for the 11,000-fragment to originate from anywhere along the HC until quite

Table 5. Amino acid composition of myosin HC and its fragments expressed as residues per 10^5 g

	90,000 dalton fragment	Head HC calculated from lit.	CNBr-fragment containing thiol-1 and thiol-2 calc. from sequence	11,000 dalton fragment	Myosin rod [31] = light meromyosin and subfragment-2
Lys	81	80	86	60	107
His	17	20	38	11	15
Arg	38	37	48	28	56
Asp	85	85	57	92	88
Thr	55	52	19	59	38
Ser	43	40	48	67	39
Glu	117	122	105	92	219
Pro	37	35	19	43	0
Gly	58	55	76	82	20
Ala	72	67	48	84	85
Cys	10	11	19	21	5
Val	51	53	48	61	33
Met	26	27	10	36	23
Ile	44	52	57	35	36
Leu	73	76	114	68	99
Tyr	34	36	29	15	6
Phe	49	47	57	29	7

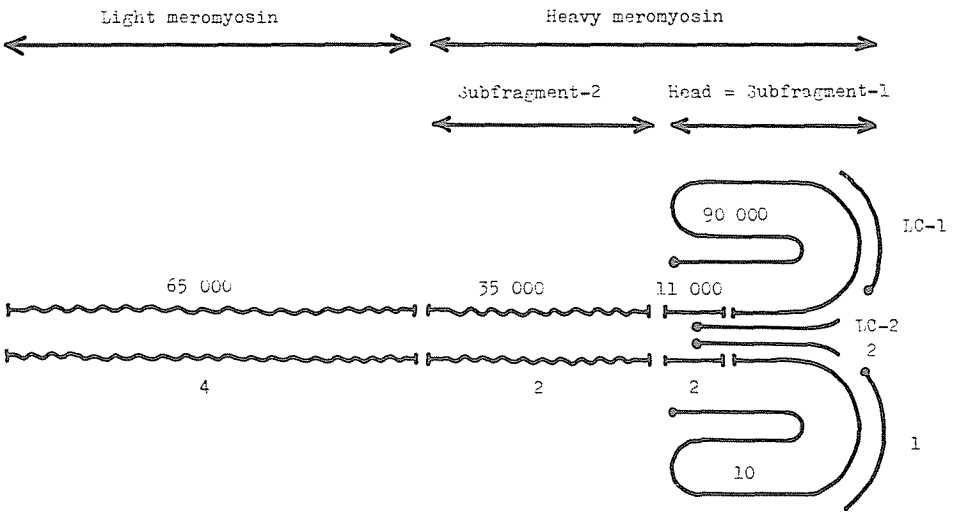


Fig. 21. Schematic representation of myosin molecule with its HC fragments and intact set of LC. Upper figures indicate molecular weights and lower figures give cysteine residues per mole of HC fragment and LC. Black dots at one end of HC and LC indicate blocked N-terminal amino groups.

close to the 2 head portions. Furthermore proline residues are incompatible with the alpha-helical conformation in proteins and the 11,000-fragment has the highest relative content of this amino acid indeed when compared with the other 2 peptides from the head region given in table 5. It thus originates from a region that probably contains very little, if at all, alpha-helical parts.

Beside the number of cysteine residues given here for the 90,000 dalton (10 per mole) and the 11,000 dalton fragment (2 per mole) the heavy meromyosin subfragment-2 is known to contain a total of 4 cysteines, i.e. 2 per chain fragment [31]. Each LC-2 contains 2 [85] and LC-1 and LC-3 contain one cysteine each [180]. This leads to a subunit distribution of cysteine residues as illustrated in fig. 21. Adding up would lead to a total of 34 cysteine residues in heavy meromyosin leaving 8 of the total amount of 42 per myosin to be assigned to the light meromyosin, i.e. 4 per chain. Around 3 cysteine residues only were found in the light meromyosin fragment per a molecular weight of about 65,000 dalton as given in fig. 21 [184, 186] so perhaps during the proteolytic fragmentation of the parent myosin 2 cysteines might have been lost.

6. Closing Remarks

The work described herein demonstrates the potential of chemical probing of functional side chain groups in the contractile protein myosin. Progressive blockage of specific thiol groups in conjunction with the ensuing changes in enzymic properties, allowed the characterization of different conformational states. This biochemical approach enabled us to recognize structure-functional relationships under experimental conditions with the protein in its native gel state as it is in muscle, when other physico-chemical methods could not be employed.

First of all conformational changes were observed as a consequence of hydrolysis of ATP at temperatures above 15° C which disappeared at 0° C. Such temperature dependent conformational changes affecting the enzyme mechanism have recently also been reported for lysozyme [191] and ribulose-1,5-bisphosphate carboxylase [192]. In intact myosin above 15° C the long lived enzyme-product complex rendered the non-essential thiol-3 groups specifically more reactive. In turn these thiol groups are located on the myosin heavy chains in a region which can be separated from the normally functioning heads. Hence hydrolysis of ATP causes syncatalytic allosteric conformational changes in the molecule. It is of particular interest that these allosteric changes do occur in the neck region where the heads are joined to the myosin rod, because this region is thought to serve as hinge around which the heads of the cross-bridge may rotate during the contraction cycle. Segmental flexibility between the heads and the rest of the molecule has indeed been shown to occur by fluorescence depolarization [193] and saturation transfer electron paramagnetic resonance [194] techniques. In addition electron microscope studies on the structure of myosin seem also to indicate that the rod-like molecule is able to bend in that neck region [195]. These observations would well tie in with our finding that the peptide containing the thiol-3 groups isolated from this very region in the heavy chains has the highest content in proline which is characteristic for protein domains that do not contain the rigid alpha-helical conformation but may allow flexibility. Similar syncatalytic allosteric changes in the myosin heavy chains occur in myosins from fast- and slow-contracting as well as from heart muscles indicating that the functional relationship between the active centre in the heads and the neck region, i.e. at the basis of the cross-bridge formed by the heads, is the same in different types of myosin.

The second important implication of our studies is the finding that binding of Mg-

ADP or Mg-ATP induces some kind of interaction between the 2 heads of an intact myosin molecule. This head-head interaction is reflected in the reaction sequence of the essential thiol-1 and thiol-2 groups indicating that under certain conditions one head is able to influence the conformation of the other. Extensive binding studies employing various methods revealed that of the 2 moles of ADP which bind per myosin, one does so with a high affinity ($K \sim 10^6\ M^{-1}$) and the other one with a much lower affinity ($K \sim 10^4\ M^{-1}$). In contrast, just one ADP was found to bind with high affinity ($K \sim 10^6\ M^{-1}$) to isolated myosin heads [144]. From this again it was deduced that some kind of interaction must take place between the 2 heads. When one ADP binds to one myosin head a negative cooperativity becomes operative leading to a lower affinity for the second ADP to the active centre of head number 2. Even in the presence of actin within the intact myofibrillar structure, we have observed a divergence in the affinities of ADP for myosin [97]. This would suggest that the free energy needed to desorb at least one of the product molecules from a myosin cross-bridge is relatively low, in any case much lower than that needed to dissociate ADP from isolated myosin heads. Thus one may speculate on the basis of the cooperativity found in intact myosin, that in a working cross-bridge the heads do function together in a concerted way. When at the beginning of the contraction cycle the product-loaded heads of the cross-bridge interact with actin, that head whose ADP is bound with lower affinity, may perform its rotational movement which is connected with the desorption of the product, slightly ahead of the other, thus leading the way.

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