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CHEMICAL AND CONFORMATIONAL EVENTS IN REGIONS OF THE MYOSIN

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Structural changes in myosin may take place within the heavy chains, or may represent rearrangement of subunits. Changes induced by ATP and possibly restricted to the heavy chains are indicated by several different optical studies, including changes in absorbence of aromatic residues (1), changes in the fluorescence of dyes coupled to myosin (2), changes in electron spin resonance spectrum (3), and changes in the intrinsic fluorescence of tryptophan residues (4, 5). A calcium dependent subunit rearrangement in scallop myosin is indicated by fluorescence depolarization studies (Cheung and Szent-Györgyi, unpublished observations). In scallop muscles, calcium regulates contraction by directly reacting with myosin (6), and the changes in the quaternary structure of myosin are likely to be related in the way calcium control works.

At first I will discuss the changes of fluorescence that Drs. Weber, Fasman, and I have described several years ago (4). Then I will speculate on the implications of a subunit rearrangement on the mechanism of regulatory light chain function.

CHANGES IN THE INTRINSIC FLUORESCENCE OF MYOSIN INDUCED BY ATP

ATP increases the fluorescence of some tryptophan residues of rabbit myosin. Since the fluorescence of heavy meromyosin (HMM) obtained by digestion with trypsin and of subfragment 1 (S1) obtained by digestion with papain (7) also increases, the tryptophan residues responding to ATP must be located in the head region of the myosin molecule. The overall enhancement of fluorescence may amount to about 17% in the case of HMM at room temperature. The maximal fluorescence enhancement requires the presence of divalent cations, and also depends on the structure of the nucleotide. The structural requirements include the presence of an amino group on position 6 of the base ring, a triphosphate moiety, and the ability of myosin to cleave the phosphate at the α-position.

The magnesium salts of dATP, adenosine tetraphosphate (ATPP), and CTP all satisfy these criteria and induce the same fluorescence change as ATP (Table I). ATP analogs that bind to HMM but are not split by it cause a more limited fluorescence enhancement. The increase induced by adenylylimidophosphate (AMP-PNP) is about 10%, that by adenylylmethylenediphosphonate (AMP-PCP) is about 3%. Furthermore, HMM modified by N-ethylmaleimide (NEM), a reagent that inhibits ATPase activity of HMM without interfering with its nucleotide binding (8), shows a reduced fluorescence enhancement of about 5% in the presence of ATP. It is of considerable importance that the

enhancement of fluorescence induced by ADP is considerably smaller than the one caused by ATP and amounts to about 5%.

These results show that a particular state of myosin during ATP hydrolysis is characterized by a maximally enhanced fluorescence. This highly fluorescent state cannot be formed by the end product of hydrolysis, or by simply binding the substrate in the absence of the cleaving step. The enhancement of fluorescence persists as long as substrate is hydrolyzed by myosin. These results indicate that there is an intermediate state of myosin which can be monitored by fluorescence (4).

The results on fluorescence are in full agreement with the electron spin resonance studies of Seidel and Gergely (3), who used a spin label that specifically combines with a particular sulfhydryl group involved in the ATPase activity of myosin. Their results also indicate that the ADP-myosin complex formed via ATP hydrolysis differs from the complex formed by combining myosin with free ADP. The existence of a specific intermediate state of myosin also explains the specific effects of ATP on the absorbtion spectrum of myosin (1) and its effect on the fluorescence of amino-naphthalene sulfonic acid that has been coupled to myosin (2).

The myosin intermediate that is associated with the highly fluorescent state is the dominant species during the steady state of ATP hydrolysis. With the aid of the transient kinetic methods several different intermediates have been identified (9). At room temperature, about 90% of the population is an intermediate of myosin which is formed after the hydrolytic step and still contains the products ADP and Pi, [M** (ADP, Pi)]. It is likely that this is the intermediate that is highly fluorescent.

The enhancement of fluorescence shows that the environment of at least some of the tryptophan residues has been altered by ATP and that these tryptophan residues are now in a more hydrophobic milieu. Although the changes in tryptophan fluorescence indicate some type of conformational change, it is by no means clear how extensive this change is. In fact, present evidence suggests that only a restricted region of the myosin heads is affected by ATP. Such evidence is obtained from fluorescence quenching and from comparative studies. Iodide ions quench the fluorescence of most tryptophan residues of myosin; however, those residues that react to ATP are protected from iodide (4, 10). One estimates that possibly only 2 out of the 20 tryptophan residues of HMM become inaccessible to iodide in the presence of ATP. The structural changes induced by ATP do not affect the whole molecule uniformly; the changes that are recorded by fluorescence are localized ones.

The increase in tryptophan fluorescence induced by ATP is not a general phenomenon characterizing all myosins. For instance, the fluorescence of scallop myosin is not altered by ATP within an experimental error of about 1% (Werber et al., unpublished observations). It appears that a tryptophan residue at the active site, or its altered environment, is not obligatory for the formation of the intermediate state. The presence of tryptophan near the active site and the changes in its fluorescence in rabbit are happy accidents that reveal some aspects of the changes taking place in the head region of myosin during ATP hydrolysis and help in the identification of the intermediate states. An alternative explanation for the lack of fluorescence in scallop myosin would assume that the mechanism of ATP hydrolysis in scallop differs fundamentally from rabbit. This, however, is an unlikely possibility.

TABLE I. Effect of Various Substrates, Analogs, and Modification of SH Groups on the Fluorescence of HMM

Material	Analog	$^{\Delta I}/1\%$ at 337 nm
Untreated HMM	ATP	17.3
Untreated HMM	dATP	17.3
Untreated HMM	ATPP	17.6
Untreated HMM	ADP	5.4
Untreated HMM	AMP	0
Untreated HMM	AMP-PCP	2.8
Untreated HMM	AMP-PNP	10.0
Untreated HMM	ITP	10.4
Untreated HMM	IDP	0
Untreated HMM	CTP	18.0
Untreated HMM	UTP	4.5
Untreated HMM	GTP	4.0
Untreated HMM	PP_i	1.6
HMM treated with mersalylic acid	ATP	0
HMM treated with n-ethylmaleimide	ATP	5

From Werber et al. (4), where conditions of measurements and the preparations are described in detail.

In rabbit myosin, changes in tryptophan fluorescence reflect and signal indirectly the conformations characterizing the various intermediates that arise from the interaction of ATP with myosin. It is not clear at present, however, how these intermediates relate to various crossbridge configuration and to the steps that generate movement and tension.

SUBUNIT INTERACTIONS IN MYOSIN

Myosin molecules consist of two heavy chains of about 200,000 daltons each and of four light chains in general of two different kinds with chain weights of less than 30,000. Recently my colleagues, John Kendrick-Jones, William Lehman, Eva M. Szentkiralyi, and I have been able to establish that a particular light chain regulates the actin-activated ATPase of some myosins. We observed that in molluskan muscles calcium regulation is linked to myosin and not to thin filaments (11). Molluskan thin filaments do not bind calcium and lack in troponin (11–13). Molluskan myosins differ from rabbit myosin. These myosins bind calcium with a high affinity in the presence of a large excess of magnesium, and, when combined with pure rabbit actin, the ATPase activity of the actomyosin complex requires calcium (11, 14, 6); (Table II). Calcium regulation of molluskan actomyosin does not require tropomyosin or troponin. Control takes place on myosin in a way that it is unable to combine with actin in the absence of calcium. Calcium reverses this inhibition by directly interacting with myosin. Myosin control may be contrasted to actin control, studied in detail in vertebrate muscles. Actin is controlled by the troponin-tropomyosin system which, in the absence of calcium, prevents its combination with myosin. Calcium reverses this inhibition by reacting with troponin.

Myosin-linked calcium regulation was most extensively studied in the scallop. Scallop myosin is very similar in its subunit structure to other myosins. It contains two heavy chains of somewhat below 200,000 daltons and four light chains of two kinds with chain

TABLE II. The Effect of the Regulatory Light Chain on the Actin-Activated ATPase Activity and On the Calcium Binding of Scallop Myosin

	Actin-activated ATPase (μmoles ATP/mg/min at 23°C)		Ratio	μmoles Ca/g myosin
	0.1 mM Ca^{2+}	0.1 mM EGTA	EGTA/Ca^{2+}	at 5 × 10^{-7} M Ca^{2+}
Placopecten				
Untreated myosin	0.23	0.01	0.04	1.54
Myosin "desensitized" by two successive EDTA treatments	0.24	0.21	0.87	0.72
"Desensitized" myosin recombined with EDTA-light chain in original proportions	0.23	0.06	0.26	1.39
"Desensitized" myosin recombined with EDTA-light chain in twice the original proportions	0.26	0.02	0.08	1.36
EDTA-light chain	none	none		none
Aequipecten				
Untreated myosin	0.22	0.05	0.23	1.67
"Desensitized" myosin	0.24	0.20	0.83	0.78
"Desensitized" myosin recombined with EDTA-light chain in original proportions	0.22	0.07	0.32	1.59
EDTA-light chain	none	none		none

ATPase activity of scallop myosin premixed with rabbit actin was measured at pH 7.5 in 30 mM NaCl, 1 mM $MgCl_2$ and 0.5 mM ATP in a pH-stat. No measurable ATPase activity was observed by testing 1 mg EDTA-light chain in the presence of rabbit actin or in 10 mM $CaCl_2$; Calcium binding could not be detected at 5 × 10^{-7} –3 × 10^{-6} M Ca^{2+} by a 0.5–1.0 mg/ml EDTA-light chain solution using equilibrium dialysis.
From Szent-Györgyi et al. (6), where the conditions for the removal of the EDTA-light chain and its recombination with "desensitized" myosin are described in detail.

weights of about 18,000 daltons. Scallop myosin forms bipolar filaments, indistinguishable from those of vertebrate myosins, at low ionic strengths, and has a globular head region and a helical rod portion. Trypsin and papain digestion yields meromyosins, S1, and rods of size and properties similar to the fragments obtained from rabbit myosin. The significant difference between scallop and rabbit myosin is found in the calcium binding and calcium sensitivity of the ATPase activity. One of the light chains can be detached from scallop myosin in the absence of divalent cations by exposing myosin preparations or myofibrils to 1–10 mM EDTA. Such preparations lose their calcium sensitivity and have a reduced calcium binding due to losses in calcium binding sites (Table II). The EDTA-treated myosin preparation is desensitized, since its activity is no longer suppressed in the absence of calcium (Table II). The scallop myosin stripped of its regulatory light chain behaves

similarly to rabbit myosin. Calcium sensitivity and calcium binding are fully regained if the regulatory light chain is readded to stripped myosin preparations in the presence of divalent cations, although the isolated light chain has no ATPase activity and binds no calcium. The recombined myosin behaves like an untreated one. The dependence of regulation on the presence of a particular light chain indicates that this light chain has a regulatory function and is a regulatory subunit (6).

The function of this light chain is to suppress ATPase activity by blocking sites on myosin required for complexing with actin in the absence of calcium. This inhibition is reversed by calcium ions at concentrations as low as 10^{-6} M. The problem is how the regulatory light chain influences actin binding sites on the globular regions of the heavy chain, and precisely what the changes are when myosin is switched from the "off" state to the "on" state. Some of the clues include evidences for cooperativity between the two myosin heads and a calcium-dependent change between light and heavy chain interactions.

Although there are two regulatory light chains in a myosin molecule, removal of one fully desensitizes myosin. Readdition of 1 mole of regulatory light chain to 1 mole of desensitized myosin fully restores regulation. Both regulatory light chains are necessary for control, and there is a cooperativity in maintaining the off state in the absence of calcium. These results however, do not suggest a cooperativity for ATPase activity in the presence of calcium. This interpretation is supported by the behavior of the proteolytic fragments of myosin. Heavy meromyosin, which contains both myosin heads connected by a short tail, has calcium sensitivity; the S1 subfragment, which is a single-headed species, shows no calcium sensitivity although it binds calcium and contains regulatory light chains. S1 does not show calcium sensitivity even in the presence of excess regulatory light chains obtained from intact myosin. A simple model of the off state would assume that in the absence of calcium the actin binding sites of myosin are blocked, and that this state requires coupling of the myosin heads via the regulatory light chains. In the on state the light chains are fixed by calcium in a nonblocking position. This type of model would predict a calcium-dependent change in the relative position of the regulatory light chains and the heavy chains.

Collaborative studies with Dr. Herbert Cheung of the University of Alabama indicate that these speculations may not be completely without foundation. We have approached the problem with the technique of fluorescent depolarization. The isolated light chains were complexed with diethylaminonaphthalene-sulfonylchloride and then recombined with desensitized myosin preparations. Correlation times were obtained on the free light chains and on the light chains that were recombined with desensitized myosins, both in the absence and in the presence of calcium ions. The three correlation times obtained were 30 nsec for the free regulatory light chain, about 250 nsec for the light chain that was recombined with myosin in the absence of calcium, and about 375–400 nsec for the recombined preparation in the presence of calcium. Several different preparations give similarly consistent results, though the absolute values varied somewhat from preparation to preparation. We draw the following tentative conclusions from these results. The light chain has a considerable asymmetry, since its correlation time is longer than expected for a spherical molecule that has a molecular weight of 18,000 daltons. Independent hydrodynamic studies of Walter F. Stafford III in my laboratory also indicate the assymetry of the regulatory light chain with an axial ratio approaching about 1:10. The increase in

correlation time when the light chain is recombined to myosin indicates a rather firm attachment of the light chain, especially in the presence of calcium, since the correlation time is similar to the value one gets by directly labeling the heavy chains of myosin. When calcium is removed by EGTA, the correlation time decreases. The results suggest that the mobility of the light chain is greater in the absence of calcium. One may speculate that calcium is able to fix the light chain in the on state, and that in the absence of calcium it is in its natural inhibitory position. This picture is obviously an oversimplification. It is not clear as yet how the cooperativity works and what is the nature of the proposed calcium-dependent rearrangement of the myosin subunits. It is of interest that such a movement of the subunits, if it takes place, would be required for the preparation of crossbridge formation and would not necessarily be part of the steps responsible for tension generation or motion.

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