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Regulation of contraction by calcium binding myosins

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Abstract

Contraction of molluscan muscles is triggered by binding of Ca^{2+} to myosin. Molluscan myosins are regulated molecules, their light chains serve as regulatory subunits. They differ from myosins of skeletal muscles in requiring Ca^{2+} for activity and having a specific high-affinity Ca^{2+} binding site. As all conventional myosins molluscan myosins also consist of two heavy chains, two regulatory and two essential light chains. Scallop myosin is particularly suitable for studying light chain function since its regulatory light chains readily dissociate in the absence of divalent cations and its essential light chains can be exchanged with foreign light chains. The structural, mutational and biochemical studies presented here are aimed to elucidate the role of the light chains in regulation, to describe the interactions between the myosin subunits and to locate the regions and the amino acids responsible for the differences between functional and non-functional light chains.

Keywords: Myosin; Molluscan muscles; Calcium binding; Contraction; Muscles

1. Personal notes

When I was young and even more naive than now, I thought that the meromyosins obtained by short tryptic digestion of myosin were subunits of myosin since their properties were entirely different. It did not take long for Bill to demonstrate with Mihalyi how wrong this interpretation was, that the formation of the meromyosins was due to the proteolysis of a particularly sensitive region in the molecule [1]. This was the birth of the "hinge region" of myosin which played such a central role in Bill's contraction model. While the theory suffered the fate of most theories, it led Bill to design a number of beautiful experiments characterizing this portion of the myosin molecule demonstrating that it could play an important role in the mechanical properties of the myosin filament, although it is not the force producing site.

It was always a pleasure to talk to Bill. He was stimulated by problems and excited by progress. I recall his delight when he heard that the functionally different myosins of striated and of catch muscle differ in the hinge region arising from the splicing of a single gene [2]. He had a warm personality and was a loyal friend. He had an immense store of curiosity and enthusiasm for science. We miss him greatly.

2. Regulation by molluscan myosins

The fascination of the molluscan regulatory system lies in its simplicity. Contraction is triggered by binding of calcium to myosin. The molecule that is

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capable to generate motion can also regulate its own activity. The structural changes induced by binding a simple ligand lead to the activation of its enzymatic machinery and to the ability to interact with actin resulting in movement and force generation. Molluscan myosins are, therefore, particularly suitable for defining the conformations of the molecule at rest, in activity and rigor by direct structural determination.

The discovery of myosin-linked regulation was accidental. We intended to analyze the properties of native thin filaments and chose to isolate these from molluscan muscles since molluscan filaments are very large. We have succeeded to obtain thin filaments without exposure to high salt concentrations or organic solvents. The filaments readily hybridized with rabbit myosin and activated its ATPase, however, to our surprise the ATPase activation was independent of the presence or absence of Ca^{2+} [3]. Evidently molluscan thin filaments were not regulated, did not bind Ca^{2+}, in contrast to the native thin filaments of skeletal muscles. We have also found that molluscan myosins differed from myosins of skeletal muscles in having a calcium sensitive actin-activated ATPase even in the absence of tropomyosin and troponin and contained a high-affinity specific calcium binding site [4]. Subsequent studies focused on the role of the light chains in regulating myosin (reviewed in Refs. [5,6]). Molluscan myosin, like their skeletal counterparts, consist of two heavy chains, two regulatory light chains (RLC) and two essential light chains (ELC). Both light chains belong to the family of Ca^{2+} binding proteins, contain four putative EF hand motifs [7,8] and are obvious candidates to function as regulatory subunits.

3. RLC functions

Scallop myosin was the choice for detailed studies since its RLCs could be reversibly detached in the absence of divalent cations [9]. At 4°C one of the two RLCs are removed from myosin, at room temperature both RLCs dissociate [10] (Fig. 1). Regulated myosin requires Ca^{2+} for the actin-activated ATPase; in the absence of Ca^{2+} activity is very low [4]. The role of the RLC is to inhibit activity in the absence but not in the presence of Ca^{2+}. Scallop

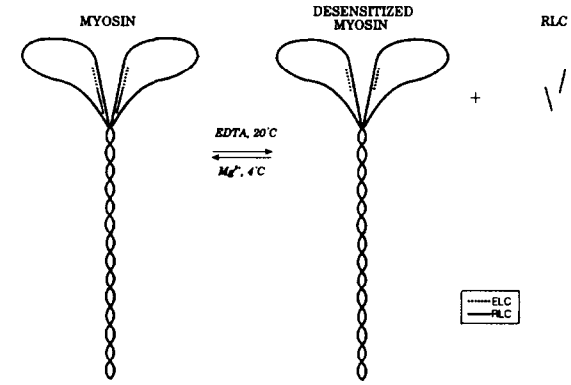

Fig. 1. Scheme of dissociation of regulatory light chains from scallop myosin with 10 mM EDTA at 23°C.

myosin from which RLC had been removed is constitutively in the "on" state, behaving similarly to skeletal myosins. Actin-activated ATPase is a good measure of regulatory function: tension generation of permeabilized fibers [11] and motility of myosin [12] have a similar Ca^{2+} concentration dependence as ATPase activity [13] and also require the presence of RLCs. The RLCs are also needed for Ca^{2+} binding even though the isolated RLCs do not bind Ca^{2+}. Ca^{2+} binding and sensitivity of ATPase activity is fully restored upon RLC rebinding. Scallop myosins deprived from their own RLCs hybridize readily with foreign RLCs. However, foreign RLCs differ in their effects. Regulatory functions are fully regained if the source of the RLCs is a regulated myosin, i.e. molluscan, smooth muscle or non-muscle myosins. RLCs of vertebrate skeletal muscles do not restore Ca^{2+} binding [14] and ATPase activity remains inhibited both in the presence and the absence of Ca^{2+} [15].

4. ELC functions

We have not succeeded to remove ELCs from scallop myosin without denaturing the heavy chain, therefore, the clarification of its role in regulation is more indirect. Foreign ELCs may be introduced into myosin by exchange, however, exchange is greatly dependent on affinities and in most cases is limited [16]. The first evidence that the ELC is also a regulatory subunit was the demonstration that poly-

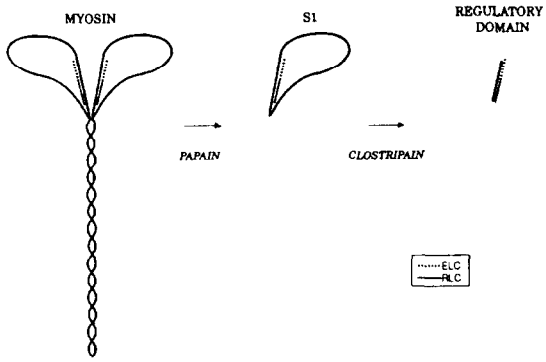

Fig. 2. Scheme of formation of scallop RD. Subfragment-1 prepared with papain is further digested with clostripain [21]. The RLC of subfragment is nicked and lacks 11 residues from the N-terminus. The heavy chain fragment begins with Ala-764 and ends at Ala-840 or Arg-841. Its weight is 9247 Da.

clonal antibodies, specific against ELC, interfered with the regulatory process by removing the inhibition in the absence of Ca^{2+} [17]. Sequence studies suggested that the ELC may contain the triggering Ca^{2+} binding site since domain III of the ELC has the liganding side-chains at the positions characteristic to EF hands of Ca^{2+} binding proteins [18].

A major advance in establishing the role of ELC was the isolation of the regulatory domain (RD) from scallop myosin [19]. The RD consists of an about 10 kDa heavy chain fragment and the two light chains. It corresponds to the neck region of subfragment-1 (S1) and is resistant to proteolysis (Fig. 2). The RD retains the specific high-affinity Ca^{2+} binding site [19,20]. Its components can be isolated in chaotropic solvents, like urea or guanidine · HCl. The RD can be reconstituted in the absence of denaturants and its Ca^{2+} binding is restored [21]. RD is readily assembled with foreign ELCs. The hybrid RDs bind Ca^{2+} only if the source of ELC is a Ca^{2+} binding myosin [21,22]. Although molluscan ELCs restore Ca^{2+} binding when complexed with the RLC and the heavy chain fragment, they are unable to bind Ca^{2+} when isolated. The results indicate that the ELC contain the triggering Ca^{2+} binding site.

5. Role of the heavy chain

Rabbit skeletal and chicken gizzard RDs are unable to bind Ca^{2+} similarly to the parent myosins. However, the heavy chain fragments hybridized with functional RLCs and ELCs show Ca^{2+} binding, although with a lesser specificity and affinity, particularly in the case of the rabbit heavy chain construct. Ca^{2+} binding, therefore, depends primarily on the light chains but is modified by the heavy chains [23]. Although the heavy chain of scallop myosin (*Argopecten irradians*) has been sequenced and the residues conserved in regulated myosins have been identified [24], their contribution to regulation is not known.

The RD has been crystallized and its structure solved at 2.8 Å resolution [25] (Fig. 3). The overall structure of the RD is similar to the corresponding region of the chicken S1 [26]. The two light chains interact seriatim with the heavy chain with an opposite polarity to it. The heavy chain forms a long α-helix that has an about 40° bend in the middle where the two light chains meet and a sharp hook near the C-terminus where the N-terminal lobe of the RLC grips the heavy chain. The structure describes in detail a network of side-chain interactions positioning the light chains to the IQ motif (IQxxxRGxxR) of the heavy chain. The structure indicates the presence of two divalent cation binding sites, one in domain I of the RLC and one in domain I of the ELC [25].

6. Functional and non-functional light chains

Ca^{2+} binding and sensitivity both require the presence of all the three components of scallop myosin, the RLC, ELC and the heavy chain. Furthermore, these functions are restored only by RLCs of regulated myosins and by ELCs of Ca^{2+} binding myosins. We followed the following strategy to pinpoint the reasons for the differences between functional and non-functional light chains: (1) Construct chimeras between functional and non-functional light chains to determine if differences between them could be traced to a single domain. (2) Mutate those residues of that domain which are conserved among functional light chains to the residues of non-functional ones to see which amino acids are necessary for function. (3) Attempt "gain in function" mutations by converting residues of the non-functional light chains into those present in scallop.

7. ELC mutations

We thought that we could make short cuts in the studies of the ELC and start with single site mutations, since the sequence pointed so convincingly to domain III as the EF hand responsible for the triggering Ca^{2+} binding site [18]. The EF hand loop of domain III in molluscs contains a serine at the $-X$ position (Ser-102 in scallop) which is methionine in vertebrate and leucine in *Drosophila* ELCs. To our surprise a $S_{102}M$ mutation had no effect on Ca^{2+} binding. Substitution of alanine for aspartate at the $+X$ position ($D_{94}A$) also failed to abolish Ca^{2+} binding although such mutation destroys divalent cation binding by EF hands [27]. We than constructed chimeras between cardiac and scallop ELCs. The important domain turned out to be domain I. Chimeras containing scallop domain I restored Ca^{2+} binding to RDs. In contrast, RDs reconstituted with chimeras containing cardiac domain I did not bind Ca^{2+}. Ca^{2+} sensitivity of ATPase activity also required an ELC with scallop domain I (Fig. 4). These results were unexpected since the loop of domain I does not have the canonical sequence characteristic of the EF hand, although it contains a cluster of molluscan specific residues (FWDGR). However, we had no easy way to determine the liganding residues by mutational studies, some of which could possibly be contributed by other segments of the ELC or even by the RLC. It was at this time that the structure was solved not only indicating the presence of Ca^{2+} in domain I but also identifying the liganding residues of this unusual Ca^{2+} binding site. A 9-residue pep-

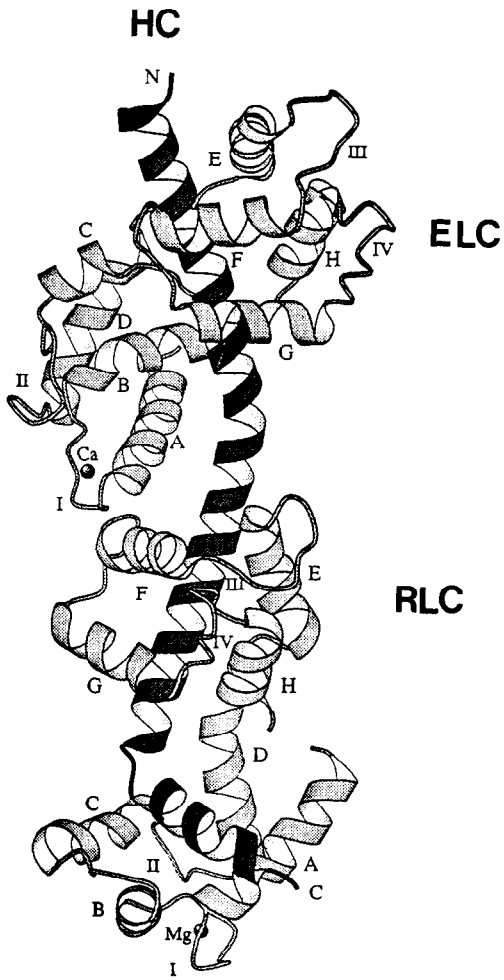

Fig. 3. Ribbon structure of scallop regulatory domain. HC, heavy chain (dark shading); RLC, regulatory light chain; ELC, essential light chain. Roman numerals, EF hands; capital letters, EF hand helices. Reproduced with permission (Xie et al., Nature, 368 (1994) 306–312).

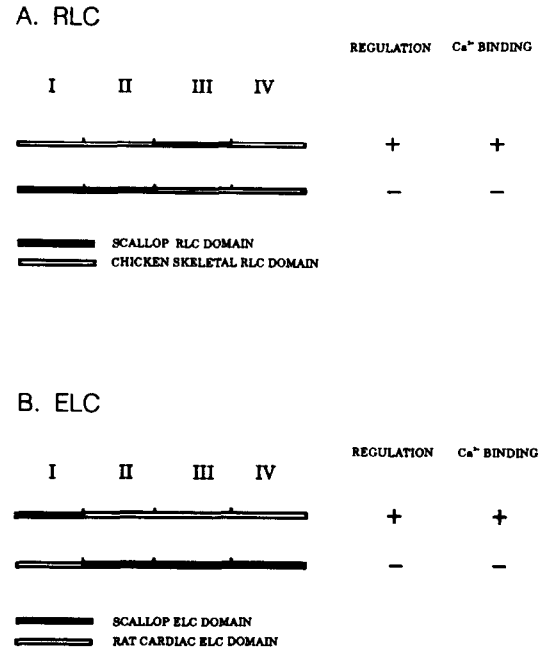

Fig. 4. Chimeras between functional and non-functional light chains. (A) Chimeras between scallop and chicken RLCs. (B) Chimeras between scallop and rat cardiac ELCs. Results show that domain I of the ELC and domain III of the RLC must be from scallop myosin for regulation and Ca^{2+} binding.

tide of the loop supplies three carboxyl oxygens (D_{19}, D_{22}, D_{25}), three main chain carbonyl oxygens (D_{19}, G_{23}, A_{27}), and a forth carbonyl oxygen which provides the seventh ligand by hydrogen bonding with an intervening water molecule (D_{22}) (Fig. 5) [25].

8. RLC mutation

Chimeras between scallop and chicken RLC restore Ca^{2+} binding and sensitivity only if the source of domain III is scallop [28] (Fig. 4). Previous studies using gizzard RLC with desensitized scallop myosin have already established that domain III of the RLC is required for Ca^{2+} binding and regulation [29]. To assess the contribution of individual residues of domain III of scallop RLCs to regulatory functions six residues were substituted with amino acids present in chicken skeletal RLC. Mutations were introduced at positions that are conserved among functional (positions 90, 116 and 117) and non-functional (positions 94, 98 and 105) RLCs. The selected residues are also characteristic for the given group and never occur in that particular position in the other one. Five of the six mutations ($R_{90}M$, $A_{94}K$, $E_{98}P$, $N_{105}K$, and $M_{116}Q$) had no effect on the regulatory properties of the scallop RLC. However, substitution of Gly-117 with alanine or cysteine had drastic consequences; the mutant RLCs lost their ability to restore the specific Ca^{2+} binding site and to confer Ca^{2+} sensitivity when added to desensitized myosin. Their affinity to the ELC–heavy chain complex has also greatly decreased [28]. The crystal structure indicates that the amino acid side-chains at positions 90, 94, 98 and 105 are solvent exposed.

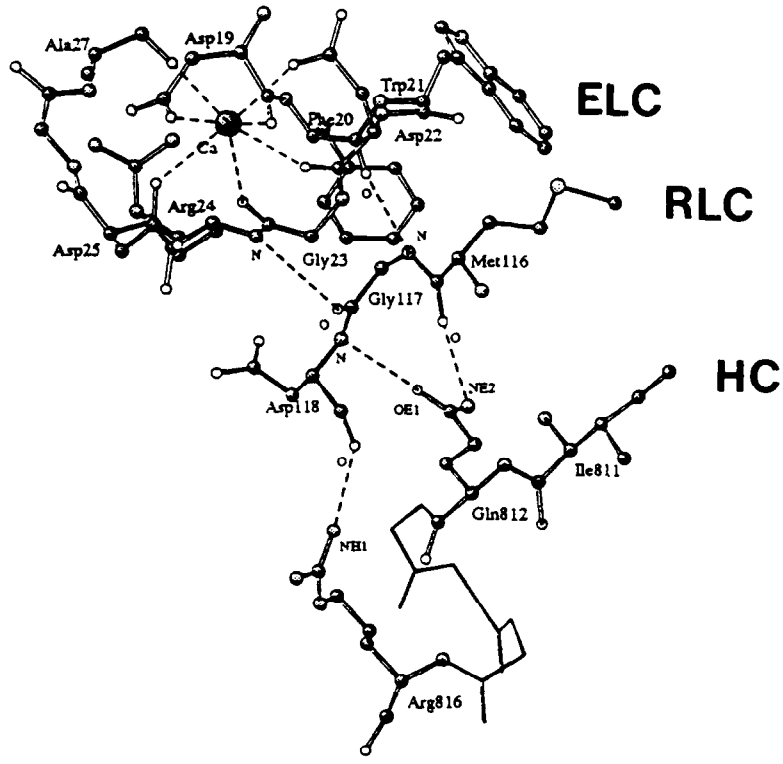

Fig. 5. Structure of the Ca^{2+} binding site of domain I of the ELC showing some of the critical linkages to the RLC and heavy chain. ELC: Asp-19 to Ala-27; RLC: Met-116 to Asp-118; heavy chain: Ile-811 to Arg-816. Reproduced with permission (Xie et al., Nature, 368 (1994) 306–312).

Met-116 forms a main-chain hydrogen bond with the heavy chain and helps to stabilize the Ca^{2+} binding site, but its side-chain is involved only in Van der Waals interactions explaining why mutation there does not affect function. Gly-117 of domain III of the RLC is in close contact with Gly-23 of domain I of the ELC and is stabilized by two hydrogen bonds: between the main-chain carbonyl oxygen of Phe-20 of the ELC and the main-chain nitrogen of Gly-117 of the RLC, and between the main-chain nitrogen of Arg-24 of the ELC and the main-chain carbonyl oxygen of Gly-117 of the RLC (Fig. 5) [25].

Gly-117 is strictly conserved among molluscan and vertebrate smooth muscle myosin RLCs. Mutation of Cys-126 of chicken skeletal myosin RLC (the residue that corresponds to Gly-117 of scallop RLC) to glycine confers to this RLC the ability to restore Ca^{2+} binding and regulation of ATPase activity, although activity is less than that of native myosin [28]. This "gain in function" mutation is the most convincing evidence supporting the key role of Gly-117 in regulation. It also demonstrates that the principal difference between functional and non-functional RLCs is the presence or absence of this crucial glycine.

9. Conclusions

The congruence of structural and mutational studies gives us some insight into the workings of myosin-linked regulation. We understand the reasons for the requirement of all the three components of myosin for regulation. Information must flow from the RD to the enzymatic and actin binding sites at some 5–7 nm distance in order to switch these functions "on" and "off". While it is likely that this communication is mediated by the long heavy chain helix, the way how it takes place is unknown. The structure of the unusual Ca^{2+} free state has not yet been determined. We are ignorant what the atomic structure of a regulated myosin molecule or a regulated myosin fragment is. There is some hope, however, that combination of structural, mutational and biochemical studies will help to pave the way for a more precise understanding of the working of the system. I am sure Bill would have enjoyed discussing this.

Acknowledgements

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