Eur. J. Biochem. **261**, 452–458 (1999) © FEBS 1999

Fluorescence measurements detect changes in scallop myosin regulatory domain

András Málnási-Csizmadia[1], György Hegyi[1], Ferenc Tölgyesi[2], Andrew G. Szent-Györgyi[3] and László Nyitray[1]

[1]*Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary;* [2]*Institute of Biophysics, Semmelweis University of Medicine, Budapest, Hungary;* [3]*Department of Biology, Brandeis University, Waltham, MA, USA*

Ca^{2+}-induced conformational changes of scallop myosin regulatory domain (RD) were studied using intrinsic fluorescence. Both the intensity and anisotropy of tryptophan fluorescence decreased significantly upon removal of Ca^{2+}. By making a mutant RD we found that the Ca^{2+}-induced fluorescence change is due mainly to Trp21 of the essential light chain which is located at the unusual Ca^{2+}-binding EF-hand motif of the first domain. This result suggests that Trp21 is in a less hydrophobic and more flexible environment in the Ca^{2+}-free state, supporting a model for regulation based on the 2 Å resolution structure of scallop RD with bound Ca^{2+} [Houdusse A. and Cohen C. (1996) *Structure* **4**, 21–32]. Binding of the fluorescent probe, 8-anilinonaphthalene-1-sulphonate (ANS) to the RD senses the dissociation of the regulatory light chain (RLC) in the presence of EDTA, by energy transfer from a tryptophan cluster (Trp818, 824, 826, 827) on the heavy chain (HC). We identified a hydrophobic pentapeptide (Leu836–Ala840) at the head–rod junction which is required for the effective energy transfer and conceivably is part of the ANS-binding site. Extension of the HC component of RD towards the rod region results in a larger ANS response, presumably indicating changes in HC–RLC interactions, which might be crucial for the regulatory function of scallop myosin.

Keywords: Ca^{2+} binding; fluorescence; myosin light chains; tryptophan.

ATPase activity, *in vitro* motility and tension generation of scallop myosin are regulated by direct Ca^{2+} binding [1,2]. The triggering Ca^{2+} ion binds to the so-called regulatory domain (RD), which occupies the neck region of the myosin and consists of a segment of the myosin heavy chain (HC) and one each of the calmodulin-like regulatory light chain (RLC) and the essential light chain (ELC). The RD, whether produced by limited proteolysis or by reconstitution from their subunits, binds Ca^{2+} with the same characteristics as intact myosin [3]. Recently, X-ray crystallography has revealed the structure of this heterotrimer in the presence of Ca^{2+} ions [4,5]. The two 'dumbbell'-shaped light chains (LCs) are wrapped around the HC fragment, which is folded into a single α-helix. The specific Ca^{2+}-binding site is a unique EF-hand motif in the first loop of ELC, stabilized by an extensive network of interactions with both the HC and RLC. Based on this structure, Houdusse and Cohen [5] proposed a regulation model in which transient flexibility in the Ca^{2+}-free state permits rearrangement of the myosin domains, thus shutting off the motor activity.

Proteolytically derived scallop myosin heads (subfragment-1; S1) are unregulated, i.e. their ATPase activity is high in both the presence and absence of Ca^{2+} [6]. Similarly, S1 isolated from vertebrate smooth muscle myosin (another regulated myosin, whose activity is triggered by phosphorylation of the RLC subunit) is also unregulated. Since RD is derived from S1, its structure is generally thought to correspond to the 'on' state of myosin. However, the unusual state in all regulated myosins is the 'off' state, which appears to require elements of both myosin heads and a portion of the myosin rod. Single-headed myosin is unregulated if made from smooth muscle myosin [7] and partially regulated if derived from scallop [8]. Trybus *et al.* [9,10] demonstrated that a stable coiled-coil portion of the rod is essential to ensure full regulation of smooth muscle myosin. Computer modeling of the head/tail junction of scallop myosin also indicates that both head–head and head–tail interactions are important for regulation [11].

In order to learn more about the head–rod junction of scallop myosin we produced and characterized recombinant RDs, some of them containing HC components extended into the rod region (RD-R). Ca^{2+}-binding properties and Ca^{2+}-induced conformational changes, as well as RLC dissociation from the recombinant and proteolytic fragments of scallop myosin, were investigated using intrinsic tryptophan fluorescence and a hydrophobic fluorescence probe, respectively. Preliminary reports of this work have been published previously [12].

Correspondence to L. Nyitray, Department of Biochemistry, Eötvös Loránd University, Puskin u. 3., H-1088 Budapest, Hungary. Fax: + 36 1 266 7830; Tel: + 36 1 266 7858; E-mail: nyitray@cerberus.elte.hu.

Abbreviations: ANS, 8-anilinonaphthalene-1-sulphonate; ELC, essential light chain; HC, heavy chain; HMM, heavy meromyosin; LC, light chain; RD, regulatory domain; RD-R, regulatory domain-rod construct; RLC, regulatory light chain; S1, myosin subfragment-1.

(Received 30 September 1998, revised 26 January 1999, accepted 28 January 1999)

MATERIALS AND METHODS

Recombinant DNA constructs

HC constructs. cDNA fragments of scallop myosin HC, coding for the neck region and various lengths of the rod were cloned into expression vectors to produce the HC component of sRD (Thr758–Pro835), mRD-R (Asp749–Arg922) and hRD-R (Asp749–Leu1175). Cloning sites and the 3′ stop signals were introduced by PCR using a scallop myosin HC clone [13] as

template. All inserts of the HC fragments, except that of sRD, were subcloned into the pET15b (Novagen) vector between *Nde*I and *Bam*HI sites. The expression vector sequence introduced a HisTag fusion peptide at the N-terminus of the constructs. The HC of sRD was subcloned into the pET5a (Novagen) vector between *Bam*HI and *Eco*RI sites, to produce a recombinant polypeptide with an N-terminal MASMTGGQQMGRG fusion sequence from the vector.

ELC constructs. Scallop wild-type [14] and a mutant (Trp21Thr) ELC were subcloned into a unique *Nde*I site of pMW172 [15] to produce a nonfusion protein. Site-directed mutation was introduced by PCR using the megaprimer method [16]. All plasmid constructs were analyzed by DNA sequencing with Sequenase, version 2.0 (Amersham).

Protein expression, purification and RD reconstitution

Recombinant DNA constructs were transformed into *Escherichia coli* BL21(DE3) pLysS cells. A single colony was inoculated into 1 L 2YT medium containing 100 $mg \cdot L^{-1}$ ampicillin. Cultures were incubated with shaking at 37 °C until the A_{600} reached 0.5–0.7 and then induced with 1 mM isopropyl thio-β-D-galactoside (IPTG). After 4 h incubation, the cells were harvested, resuspended in 8 M GuHCl, 0.5 M NaCl and 20 $\mu g \cdot mL^{-1}$ leupeptin, sonicated to disrupt DNA and cleared by centrifugation. The pH of the supernatant was adjusted to 7.5 with 20 mM Tris/HCl and loaded onto equilibrated His-Bind Resin (Novagen) or ProBond (Invitrogen) nickel-chelating columns. After washing the column with 8 M urea, 0.5 M NaCl, 20 mM Tris/HCl pH 5.3, the target protein was eluted by decreasing the pH to 4.0. Alternatively, HC of sRD was loaded onto a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) in 8 M GuHCl, 0.1% trifluoroacetic acid, 10% acetonitrile, washed with 30% acetonitrile and 0.1% trifluoroacetic acid, and eluted with 40% acetonitrile, 0.1% trifluoroacetic acid.

RD-Rs were reconstituted by mixing a stoichiometric amount of recombinant HC fragments with both LCs in 6 M GuHCl, 10 mM dithiothreitol, 20 mM Tris/HCl pH 7.5, dialyzing overnight against renaturation buffer (40 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM dithiothreitol, 20 mM Tris/HCl, pH 7.5) in 6 M urea, then continuously decreasing the urea concentration to 0 M over 24 h. Protein precipitate was removed by centrifugation and the reconstituted RD-Rs, sRD was purified either on MonoQ HR 5/5 (Pharmacia) or Superdex 200 HR 10/30 (Pharmacia) columns equilibrated with renaturation buffer.

ELC was expressed in *E. coli* and purified as described for scallop RLC [17]. Samples were further purified by reverse-phase chromatography (ProRPC).

Scallop myosin and papain S1 were prepared according to Stafford *et al*. [18]. Scallop heavy meromyosin (HMM) was prepared according to Kalabokis and Szent-Györgyi [19]. Scallop proteolytic RD (pRD) was prepared by digesting myosin with papain as described by Kwon *et al*. [3]. Light chains were isolated according to Kendrick-Jones *et al*. [20].

Ca^{2+}-binding assay

Ca^{2+} binding of sRD and RD-Rs was measured at 4 °C using equilibrium dialysis in a medium containing 20 mM NaCl, 20 mM Hepes pH 7.6, 2 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.11 mM $CaCl_2$ (0.2 $\mu Ci \cdot mL^{-1}$) and an appropriate amount of EGTA to obtain the desired free Ca^{2+} concentration as described previously [21].

Analytical gel filtration assays

Superdex 200 HR 10/30 and Superdex 75 HR 10/30 (Pharmacia) FPLC gel exclusion columns were equilibrated with 40 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$ or 0.5 mM EGTA, 20 mM Tris/HCl (or 10 mM Hepes) pH 7.5; 50–100 μL of 0.5–1 $mg \cdot mL^{-1}$ protein was loaded per run, and the flow rate was 0.5–0.7 $mL \cdot min^{-1}$. The elution buffer contained 0.1% isopropanol to inhibit hydrophobic interactions with the column bead and dimerization of mRD-R. The system was controlled by LKB HPLC pumps (Model 2249) providing a flow-rate reproducibility of better than 0.3%.

Fluorescence measurements

Intrinsic tryptophan fluorescence and 8-anilinonaphthalene-1-sulphonate (ANS) fluorescence measurements were performed on a Spex Flouromax photon counting instrument. The cuvette temperature was held at 20 °C by a circulating water system. RLC dissociation was followed by the coincident increase in ANS fluorescence. ANS fluorescence was induced via energy transfer from tryptophan residues ($\lambda_{em,TRP}$ = 340 nm, $\lambda_{ex,ANS}$ = 380 nm). Emission of ANS is blue-shifted in a hydrophobic environment from 515 nm to 468 nm [22]. The protein concentration was 0.2–0.4 $mg \cdot mL^{-1}$ in most experiments. The samples also contained 20 μM ANS, 10 mM Hepes pH 7.2, 40 mM NaCl, 0.1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ or 2 mM EDTA. The samples were excited at 295 nm [23]. Intrinsic fluorescence was measured in 40 mM NaCl, 2 mM $MgCl_2$, 20 mM Tris/HCl pH 7.6, 0.11 mM $CaCl_2$ and appropriate amounts of EGTA. Tryptophan residues were excited at 295 nm.

Tryptophan anisotropy measurements were performed on a CD900-type luminometer (Edinburgh Analytical Instruments) at 20 °C. Tryptophan residues were excited between 275 and 305 nm and the emission was observed at 340 nm.

RESULTS

Recombinant RDs and RD-Rs

Four different HC fragments of scallop myosin were expressed in *E. coli*. Their respective N-termini and C-termini, as well as the termini of the three proteolytic fragments used in this work, are summarized in Fig. 1. After purification of the HC fragments by Ni^{2+}-affinity chromatography and HPLC, they were reconstituted with the appropriate amount of RLC and ELC. The resulting heterotrimeric complexes were separated from the constituent peptides using MonoQ ion-exchange or gel filtration columns. The electrophoretic profile of the reconstituted complexes shows a 2 : 1 stoichiometry of LC to HC, as the intact RLC and ELC have identical mobility on SDS/PAGE (Fig. 2). Dimerization of the complexes with an extended HC subunit was studied by electron microscopy, electrophoresis and analytical gel filtration, and is described elsewhere [24].

Ca^{2+} binding is determined by intrinsic fluorescence measurement

All recombinant constructs bind Ca^{2+} specifically despite the presence of 2 mM Mg^{2+}. Using equilibrium dialysis 0.67–0.72 bound Ca^{2+}/Ca^{2+}-binding site was measured at pCa 5 in each reconstituted complex. This value is comparable with the Ca^{2+} binding of S1 and pRD, two myosin fragments produced by proteolysis (≈0.7 bound Ca^{2+}/Ca^{2+}-binding site) [2,3].

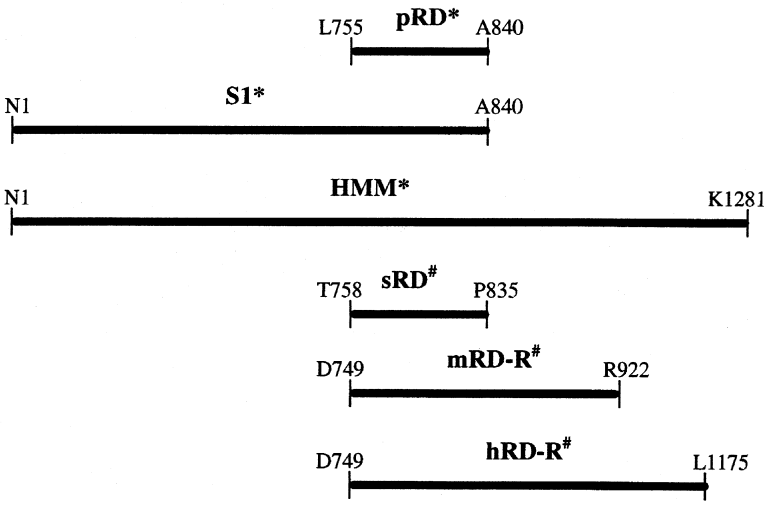

Fig. 1. Recombinant and proteolytic fragments of myosin used for the experiments. N-terminal and C-terminal residues of the appropriate myosin HC fragments, respectively, are shown. # Recombinant construct; *proteolytic fragment.

The intrinsic fluorescence of scallop myosin has been shown previously to change upon addition of either ATP or Ca^{2+} [25]. Here the relative tryptophan fluorescence intensity of the samples was measured as a function of Ca^{2+} concentration (Fig. 3A). Fluorescence intensity increases with Ca^{2+} concentration and follows a saturation curve. Half-maximal fluorescence change is at a pCa of 6.7–7.3. All complexes have very similar Ca^{2+} titration curves. None of the recombinant complexes, e.g. proteolytic pRD, bind Ca^{2+} cooperatively. By contrast, scallop myosin and HMM in the presence of nucleotide do bind Ca^{2+} cooperatively [19].

Fluorescence anisotropy of the samples also changes significantly upon the addition of Ca^{2+}. The anisotropy is smaller throughout the excitation range (275–305 nm) in the absence of Ca^{2+}. Tryptophan anisotropy values at 295 nm of mRD-R were 0.161 ± 0.001 and 0.148 ± 0.002 in the presence and absence of Ca^{2+}, respectively.

Effects the Trp21 Thr mutation of the ELC on intrinsic fluorescence

The intrinsic fluorescence of scallop RD is due primarily to their tryptophan residues. Of its five tryptophan residues, four are clustered in the RLC-binding region of the HC (Trp818, 824, 826, 827) and one is the end of the A-helix of the ELC (Trp21). The proximity of the latter residue to the Ca^{2+}-binding loop [5] suggests that it may be responsible for Ca^{2+}-dependent changes in fluorescence. Consequently, we mutated Trp21 of the ELC to threonine, then used the altered light chain to reconstitute the pRD. (ELCs of non-Ca^{2+}-binding myosins contain a threonine in a homologous position to Trp21.) The mutant construct retains the Ca^{2+} affinity of the wild-type pRD (data not shown). As shown in Fig. 3B, substitution of a nonaromatic residue at ELC position 21 greatly alters the fluorescent properties of the resulting complex. In the presence of a saturating concentration of Ca^{2+} the mutant pRD displays only 42% of the wild-type fluorescence. Furthermore, the removal of free Ca^{2+} with EGTA results in only 27% of the fluorescence change with the mutant compared with the parent molecule. These results indicate that

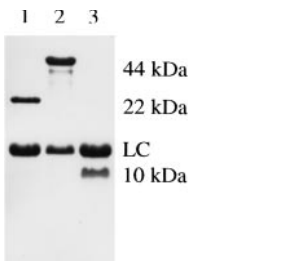

Fig. 2. Electrophoretic profile of the recombinant RDs. Reconstituted mRD-R (lane1), hRD-R (lane2) and sRD (lane3) were run on 15% SDS polyacrylamide gel. The approximate molecular weights of the RD heavy chains are indicated. RLC and ELC have the same mobility. Protein bands were visualized by Commassie brillant blue R staining.

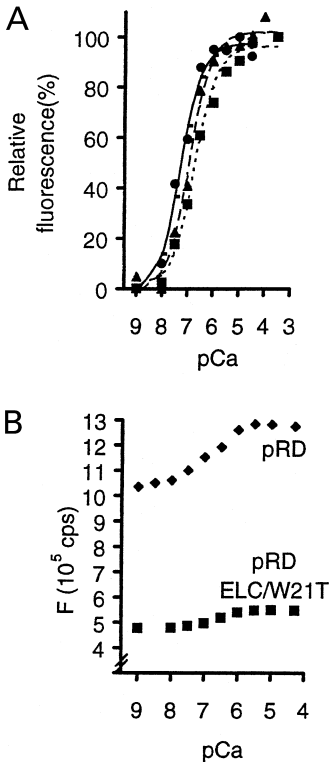

Fig. 3. Effect of Ca^{2+} on the intrinsic tryptophan fluorescence of RDs (A) and the ELC Trp21 Thr mutant pRD (B). (A) Tryptophan fluorescence of sRD (■, dotted line), mRD-R (▲, dashed line) and hRD-R (●, solid line) were measured at different free Ca^{2+} concentrations in 10 mM Mops pH 7.6, 20 mM NaCl, 2 mM $MgCl_2$, 0.11 mM $CaCl_2$ and an appropriate amount of EGTA, at 20 °C. The protein concentration was 1 μM, 100% is the maximum fluorescence, 0% is the minimum value of each curve. (B) Comparison of the intrinsic fluorescence change of wild-type and mutant pRD in which Trp21 of ELC was changed to threonine. The protein concentration was 0.8 μM.

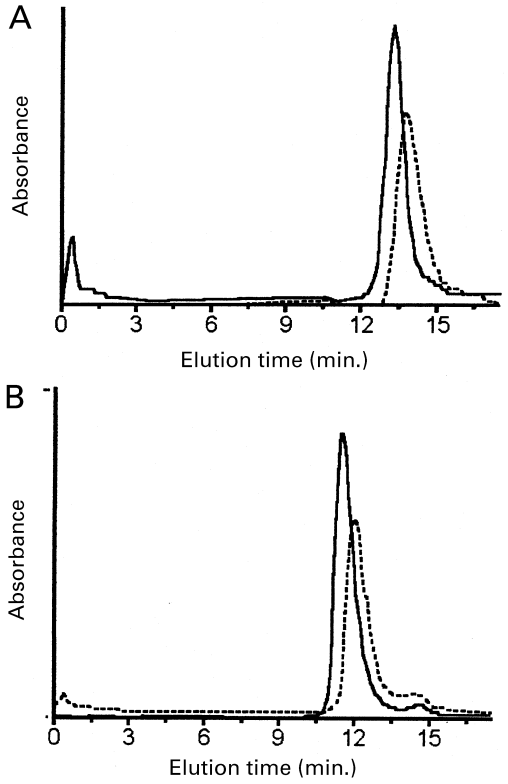

Fig. 4. Effect of Ca^{2+} on the analytical gel filtration profile of sRD (A) and mRD-R (B). Protein (0.5 mg) was loaded onto a Pharmacia Superdex 75 HR 10/30 FPLC column in the presence 40 mM NaCl, 20 mM Tris/HCl, pH 7.5, 2 mM $MgCl_2$, 0.1% isopropanol and 0.1 mM $CaCl_2$ (solid line) or 1 mM EGTA (dotted line), at a flow rate of 0.6 mL·min^{-1}.

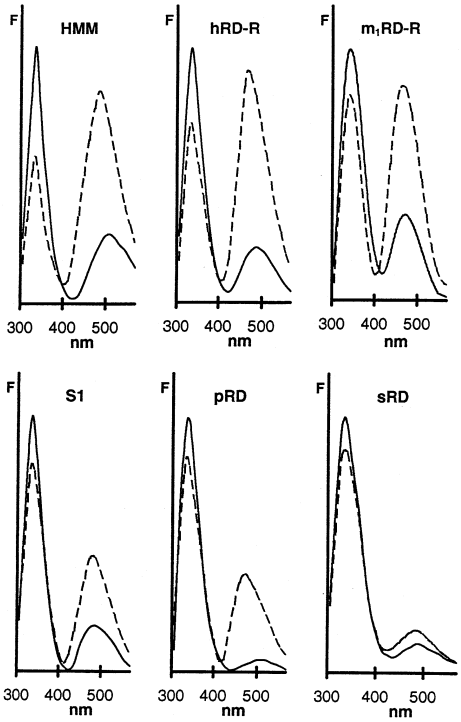

Fig. 5. Fluorescence emission spectra of ANS binding to various RDs and proteolytic fragments of myosin. Fluorescence emission spectra of HMM, hRD-R, mRD-R, S1, pRD and sRD were recorded by excitation at 295 nm at a concentration of 0.2–0.6 mg·mL^{-1}. The buffer used was 40 mM NaCl, 20 mM Mops pH 7.4, 20 μM ANS and 1 mM $MgCl_2$ (solid lines) or 4 mM EDTA (dotted lines), at 20 °C. Extensive energy transfer was observed between tryptophan residues (λ_{em} = 340 nm) and the bound ANS (λ_{ex} = 380 nm, λ_{em} = 470 nm). Ordinates show fluorescence in arbitrary units.

upon Ca^{2+} binding to ELC, the environment of Trp21 changes considerably. In addition, the four tryptophan residues in the HC of the RD are also affected, although to a lesser degree.

Ca^{2+}-induced change of RD detected by analytical gel filtration

The hydrodynamic properties of sRD and mRD-R were assayed using a Pharmacia Superdex 75 HR 10/30 FPLC gel exclusion column. As shown in Fig. 4, Ca^{2+} had a significant effect on the elution profile of each. Reducing the pCa from 4.0 to 7.0 increased the retention time by 34 ± 3 s for sRD and 29 ± 3 s for mRD-R, ≈4% in each case (at a flow rate of 0.6 mL·min^{-1}). This change indicates some compaction (smaller apparent molecular volume) of the complexes upon the removal of Ca^{2+}, irrespective of the presence or absence of the rod extension.

Dissociation of RLC studied by a hydrophobic fluorescence probe

Removal of RLC from scallop myosin, which is readily achieved by EDTA treatment, can be monitored by the water-soluble aromatic compound ANS. While weakly fluorescent in aqueous solution, emission from this compound is both shifted and enhanced when it is transferred to a hydrophobic environment [22]. In particular, ANS binds to the hydrophobic patch exposed on the surface of the HC upon dissociation of the RLC [23]. Binding of the fluorescent probe in place of the RLC can be specifically monitored by excitation at 295 nm via energy transfer from protein tryptophan residues, so only adjacent ANS molecules are energized.

To investigate this phenomenon further, we studied RLC dissociation of sRD, pRD, the rod extended mRD-R and hRD-R, as well as HMM and S1. Only HMM and hRD-R, whose heavy chain component contains over 300 residues downstream of the LC-binding domain, exist predominately as dimers. Even mRD-R, which has ≈80 residues of the rod domain, forms mostly monomeric species, i.e. heterotrimers with a HC : RLC : ELC stoichiometry of 1 : 1 : 1 [24].

Figure 5 shows the fluorescence spectra (in the presence of Mg^{2+} and EDTA, respectively) of ANS added to each of the constructs. A comparably larger enhancement of emission at 470 nm is observed when free Mg^{2+} is removed from the solution of HMM, hRD-R or mRD-R. The increased emission from ANS is accompanied by a decrease in the fluorescence of tryptophan at 340 nm, indicative of fluorescence energy transfer. Smaller changes are observed with S1 and pRD. The latter two fragments differ from the other three in their lack of residues derived from the rod domain. Dimerization does not seem to affect RLC dissociation, as mRD-R monomers exhibit the same strong fluorescence change as hRD-R dimers. Removal of Ca^{2+} by EGTA does not have any significant effect on the ANS spectra (data not shown).

The result of the ANS experiments using sRD is strikingly different: there is virtually no change in the fluorescence spectrum. It should be noted that sRD differs from pRD only in its HC component, which is shorter by five residues (LLSIA) at

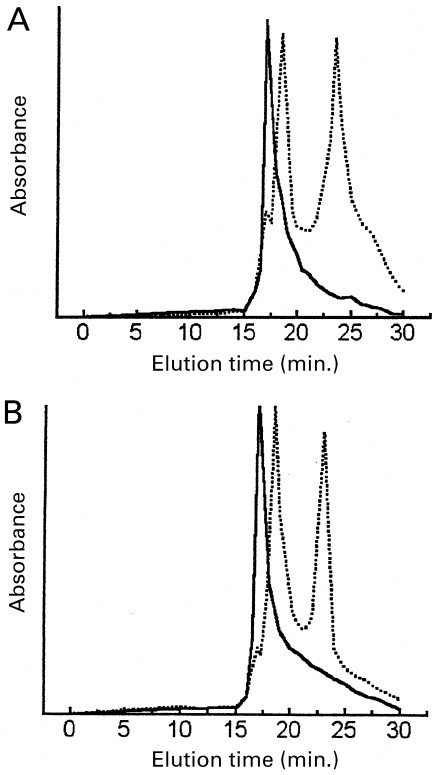

Fig. 6. Gel filtration of sRD (A) and pRD (B) in the presence and absence of free Mg^{2+}. sRD and pRD (0.5 mg) were loaded onto the Pharmacia Superdex 75 HR 10/30 column, respectively, in 100 mM NaCl, 20 mM Mops pH 7.5, 0.1 mM $CaCl_2$, 0.5% isopropanol and 2 mM $MgCl_2$ (solid lines) or 2 mM EDTA (dotted lines). In the presence of EDTA dissociated RLC appeared as a new peak, while the position of the ELC–HC complex shifted slightly from the parent sRD and pRD peaks.

the C-terminus. To determine whether this small difference causes retention of the RLC in the absence of Mg^{2+} or eliminates fluorescence energy transfer, we assayed the dissociation of sRD using analytical gel filtration (Fig. 6). A Superdex 75 gel filtration column was loaded with sRD and pRD in the presence of either Mg^{2+} or EDTA. The results indicate that removal of the divalent cation induces dissociation of RLC from both sRD and pRD. The first peak, which elutes slightly later than the intact complex, consists of the heterodimer of a HC fragment and ELC. This peak is asymmetric, as residual heterotrimers produce a shoulder on its leading edge indicating that the dissociation is not complete. The second peak is the free RLC. Thus, the anomalous behavior of sRD does not result from a nondissociable RLC.

DISCUSSION

Ca^{2+}-induced conformational change in recombinant RDs

Recombinant RD complexes can easily be produced by expressing the HC fragments in *E. coli* and reconstituting them with RLC and ELC. Two of these complexes, mRD-R and hRD-R, contain 87 and 340 residues from the rod domain, respectively, in addition to the LC-binding region. We have shown that most of the hRD-R molecules dimerize through the rod sequence, which forms an α-helical coiled-coil. However, mRD-R, the HC component that is extended by only 87 residues beyond Pro-835 (the myosin rod is thought to start downstream of this Pro), exists mostly as monomeric species [24].

Recombinant RDs, whether monomeric or dimeric, bind Ca^{2+} with a specificity and affinity similar to the parent myosin or pRD. Ca^{2+} binding induces changes in the internal mobility of RDs, as indicated by fluorescence measurements and analytical gel filtration studies. The reduced anisotropy in the absence of Ca^{2+} reveals enhanced mobility of the protein structure around tryptophan residues. The smaller Stokes-radius (an apparently less asymmetric structure), observed by gel filtration, might also be due to an increased flexibility of the Ca^{2+}-free complex. These results support a model of the Ca^{2+} switch proposed by Houdusse and Cohen [5], which requires an initial flexibility of the RD upon the loss of Ca^{2+}. In the myosin molecule this flexible state might promote new specific interactions to produce an alternative stable (off) state. However, isolated RD lacking the motor domain, does not function as a molecular switch, and it likely remains permanently in the higher mobility structure in the absence of Ca^{2+}.

Ca^{2+}-induced conformational changes were also detected by measuring the intrinsic fluorescence of RDs. Wells *et al.* [25] have previously shown that scallop myosin has a tryptophan-containing domain (the RD), which responds to Ca^{2+} binding. We were able to titrate Ca^{2+} binding of the recombinant constructs, which follows a saturation curve. Measuring tryptophan fluorescence is an easy and sensitive method for determining the Ca^{2+}-binding properties of RDs. All of the complexes studied have similar Ca^{2+}-titration curves with no cooperativity. Apparently, neither attachment to the rod nor dimerization of the RDs (as in hRD-R) affects the Ca^{2+}-binding site in the first EF-hand of ELC.

Which of the five tryptophan residues is involved in the Ca^{2+}-induced conformational changes? There are five tryptophans in the RD: four clustered in the HC, forming part of the binding site for the N-terminal lobe of RLC; and one at the beginning of the Ca^{2+}-binding loop of ELC (Trp21) [4,5]. We compared the Ca^{2+}-induced increase in fluorescence in the wild-type pRD with a mutant pRD in which Trp21 was changed to a threonine. The mutant binds Ca^{2+} with the same affinity as the wild-type complex indicating that the mutation does not significantly influence the structure. At pCa 5.0, the fluorescence intensity of the mutant RD is about half that of the wild-type pRD. The relatively low fluorescence intensity of the four tryptophans in the cluster might be due to energy transfer between these closely placed tryptophans [26]. Based on the fluorescence spectra, the Ca^{2+}-induced fluorescence change in Trp21 is considerably larger than that of the tryptophan cluster. Trp21 is located in the Ca^{2+}-specific EF-hand; its side chain interacts with the HC helix (sandwiched between Leu800 and Leu808). Here we provide functional evidence that the position of Trp21 changes upon removal of Ca^{2+}, as inferred from the Ca^{2+} bound structure [3]. Because these changes also affect the HC helix, small perturbation near the Ca^{2+} site must be propagated along the α-helix, so that the environment of the distal tryptophan cluster of the HC (roughly 30 Å from the Ca^{2+} binding loop) is also altered slightly. An alternative explanation would be to assume a more global change in the internal mobility of the whole domain upon dissociation of Ca^{2+}, which is sensed even at the remote tryptophan cluster.

HC–RLC interactions investigated by ANS fluorescence measurements

Scallop myosin is unusual among conventional myosins in that its RLC is bound relatively weakly to the HC. Its removal makes the ATPase activity of scallop myosin independent of free Ca^{2+}. Regulation is also lost if the myosin rod is proteolytically

removed. In summary, the interaction of the RLC with the HC is critical for regulation [1,2]. Therefore, detailed characterization of the interface of RLC with HC (and ELC) is essential to understanding the regulatory switch. In this paper a possibly important interaction site of the rod sequences with RLC is described, which was recognized by studying RLC dissociation with the help of a fluorescence probe.

ANS is a hydrophobic fluorophore that can be excited at either 370 nm or 295 nm via energy transfer from protein tryptophan residues [22]. Following RLC dissociation, ANS binds weakly to hydrophobic sites on the denuded HC–ELC complex and its emission increases if excited through tryptophans on the HC as shown by Bennett *et al*. [23,27]. There is a larger ANS response, indicating more extensive RLC dissociation from HMM and hRD-R, compared with S1 and pRD. Interactions between the RLC, the LC-binding region and the coiled-coil rod sequence are perturbed and/or destroyed during the proteolysis that generates S1 and pRD, consequently changing the interaction of myosin HC with RLC. The myosin rod could affect the LC-binding domain directly or by linking the two RDs into a dimer. We found that the extent of RLC dissociation was as large from the monomer mRD-R as from the dimer hRD-R and HMM. These results suggest that only a relatively short rod extension (< 100 residues) is required to attain HC–RLC interactions (as inferred from the ANS response) similar to those in the case of HMM, and that dimerization *per se* does not affect this property of the RD.

In contrast to pRD, the ANS fluorescence of sRD did not change on removal of Mg^{2+} ions. To check whether RLC dissociation occurred in both sRD and pRD, analytical gel filtration was performed with the EDTA-treated samples; the same amount of free RLC was detected in both cases indicating that RLC dissociates from sRD to the same extent as it does from pRD. The HC component of sRD is shorter by five residues than the proteolytic component [3]. The difference is due to the sequence Leu836-Leu-Ser-Ile-Ala840, which is highly hydrophobic and is very close (15–20 Å) to the tryptophan cluster (Trp818, 824, 826, 827) of the RLC-binding site on the HC. Based on these experiments we conclude that either the Leu836–Ala840 pentapeptide is a critical part of the ANS-binding site, or these residues enable another apolar segment of the HC to bind ANS. The latter segment could be around the tryptophan cluster, the 'hook' in the α-helix, which might be inaccessible to ANS in sRD. From these results it is not clear whether ANS is able to bind to site(s) on the denuded HC–ELC complex, which is too far from the tryptophan cluster to be excited by energy transfer. If the Leu836–Ala840 peptide is the only ANS site on the HC then we should assume that RLC dissociation occurs in several steps and only this site is exposed as a hydrophobic surface so that ANS is able to bind to it. The larger part of the RLC-binding site (the denuded α-helix of the HC) may collapse before it becomes accessible to ANS.

The pentapeptide is presumably buried if the molecule is complexed with RLC and interacts with the N-terminal lobe of RLC. In the crystal structure of pRD, the few C-terminal residues of the HC are not well-resolved, although Leu836 and Leu837 on the HC and Met26 and Leu47 of RLC on the lower resolution structure were shown to be in close proximity [4]. In a computer model of the scallop myosin head–tail junction, residues Leu837, Ser838 and Ala840 lie close to the A-helix of RLC [11]. This contact site between the myosin head and the beginning of the tail might be functionally important. Notable in this regard is that, if the Mg^{2+}-binding site in the N-terminal lobe of RLC (15–20 Å from the putative ANS-binding site) is destroyed by site-directed mutagenesis, affinity between the RLC and HC weakens, and the regulation of myosin is lost, although Ca^{2+} binding is not altered [28].

ACKNOWLEDGEMENTS

We thank Sylvia Fromherz for providing the ELC(W21T) DNA construct. This work was supported by grants from the US-Hungarian Science & Technology Joint Fund (JF 317) and the Hungarian Scientific Research Fund (OTKA T-023618) to L.N. and by NIH grants AR15963 and AR41803 to A.G.S.

REFERENCES

1. Szent-Györgyi, A.G. & Chantler, P.D. (1994) Control of contraction by calcium binding to myosin. In *Myology* (Engel, A.G. & Franzini-Armstrong, C., eds.), pp. 506–528. McGraw-Hill, New York.
2. Szent-Györgyi, A.G. (1996) Regulation of contraction by calcium binding myosins. *Biophys. Chem.* **59**, 357–363.
3. Kwon, H., Goodwin, E.B., Nyitray, L., Berliner, E., O'Neall-Hennessey, E., Melandri, F.D. & Szent-Györgyi, A.G. (1990) Isolation of the regulatory domain of scallop myosin: role of the essential light chain in calcium binding. *Proc. Natl Acad. Sci. USA* **87**, 4771–4775.
4. Xie, X., Harrison, D.H., Schlichting, I., Sweet, R.M., Kalabokis, V.N., Szent-Györgyi, A.G. & Cohen, C. (1994) Structure of the regulatory domain of scallop myosin at 2.8 Å resolution. *Nature* **368**, 306–312.
5. Houdusse, A. & Cohen, C. (1996) Structure of the regulatory domain of scallop myosin at 2 Å resolution: implications for regulation. *Structure* **4**, 21–32.
6. Szent-Györgyi, A.G., Szentkiralyi, E.M. & Kendrick-Jones, J. (1973) The light chains of scallop myosin as regulatory subunits. *J. Mol. Biol.* **74**, 179–203.
7. Cremo, C.R., Sellers, J.R. & Facemayer, K.C. (1995) Two heads are required for phosphorylation-dependent regulation of smooth muscle myosin. *J. Biol. Chem.* **270**, 2171–2175.
8. Kalabokis, V.N., Vibert, P., York, M.L. & Szent-Györgyi, A.G. (1996) Single-headed scallop myosin and regulation. *J. Biol. Chem.* **271**, 26779–26782.
9. Trybus, K.M. (1994) Regulation of expressed truncated smooth muscle myosins. Role of the essential light chain and tail length. *J. Biol. Chem.* **269**, 20819–20882.
10. Trybus, K.M., Freyzon, Y., Faust, L.Z. & Sweeney, H.L. (1997) Spare the rod, spoil the regulation: necessity for a myosin rod. *Proc. Natl Acad. Sci. USA.* **94**, 48–52.
11. Offer, G. & Knight, P. (1996) The structure of the head–tail junction of the myosin molecule. *J. Mol. Biol.* **256**, 407–416.
12. Málnási Csizmadia, A., Hegyi, G., Szent-Györgyi, A.G. & Nyitray, L. (1996) Characterization of recombinant scallop myosin regulatory domains containing heavy chains with various length. *J. Muscle Res. Cell Motil.* **17**, 149.
13. Nyitray, L., Goodwin, E.B. & Szent-Györgyi, A.G. (1991) Complete primary structure of a scallop striated muscle myosin heavy chain. Sequence comparison with other heavy chains reveals regions that might be critical for regulation. *J. Biol. Chem.* **266**, 18469–18476.
14. Goodwin, E.B., Szent-Györgyi, A.G. & Leinwand, L.A. (1987) Cloning and characterization of the scallop essential and regulatory myosin light chain cDNAs. *J. Biol. Chem.* **262**, 11052–11056.
15. Way, M., Pope, B., Gooch, J., Hawkins, M. & Weeds, G. (1990) Identification of a region in segment 1 of gelsolin critical for actin binding. *EMBO J.* **9**, 4103–4109.
16. Sarkar, G. & Sommer, S.S. (1990) The 'megaprimer' method of site-directed mutagenesis. *Biotechniques* **8**, 404–407.
17. Jancsó, A. & Szent-Györgyi, A.G. (1994) Regulation of scallop myosin by the regulatory light chain depends on a single glycine residue. *Proc. Natl Acad. Sci. USA* **91**, 8762–8766.
18. Stafford, W.F., Szentkirályi, E.M. & Szent-Györgyi, A.G. (1979) Regulatory properties of single-headed fragments of scallop myosin. *Biochemistry* **18**, 5273–5280.
19. Kalabokis, V.N. & Szent-Györgyi, A.G. (1997) Cooperativity and regulation of scallop myosin and myosin fragments. *Biochemistry* **36**, 15834–15840.

20. Kendrick-Jones, J., Szentkiralyi, E.M. & Szent-Györgyi, A.G. (1976) Regulatory light chains in myosins. *J. Mol. Biol.* **104**, 747–775.
21. Kalabokis, V.N., O'Neall-Hennessey, E. & Szent-Györgyi, A.G. (1994) Regulatory domains of myosins: influence of heavy chain on Ca^{2+}-binding. *J. Muscle Res. Cell Motil.* **15**, 547–553.
22. Stryer, L. (1965) The interaction of a naphthalene dye with apomyoglobin and apohemoglobin. A fluorescent probe of non-polar binding sites. *J. Mol. Biol.* **13**, 482–495.
23. Bennett, A.J., Patel, N., Wells, C. & Bagshaw, C.R. (1984) 8-Anilino-1-naphthalenesulphonate, a fluorescent probe for the regulatory light chain binding site of scallop myosin. *J. Muscle Res. Cell Motil.* **5**, 165–182.
24. Málnási-Csizmadia, A., Shimony, E., Hegyi, G., Szent-Györgyi, A.G. & Nyitray, L. (1998) Dimerization of the head–rod junction of scallop myosin. *Biochem. Biophys. Res. Commun.* **252**, 595–601.
25. Wells, C., Warriner, K.E. & Bagshaw, C.R. (1985) Fluorescence studies on the nucleotide- and Ca^{2+}-binding domains of molluscan myosin. *Biochem. J.* **231**, 31–38.
26. Eftink, M.R., Ramsay, G.D., Burns, L., Maki, A.H., Mann, C.J. & Matthews, C.R. (1993) Luminescence studies with trp repressor and its single-tryptophan mutants. *Biochemistry* **32**, 9189–9198.
27. Bennett, A.J. & Bagshaw, C.R. (1986) The mechanism of regulatory light chain dissociation from scallop myosin. *Biochem. J.* **233**, 179–186.
28. Goodwin, E.B., Leinwand, L.A. & Szent-Györgyi, A.G. (1990) Regulation of scallop myosin by mutant regulatory light chains. *J. Mol. Biol.* **216**, 85–93.