Myosin Loop 2 Is Involved in the Formation of a Trimeric Complex of Twitchin, Actin, and Myosin*

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Molluscan smooth muscles exhibit a low energy cost contraction called catch. Catch is regulated by twitchin phosphorylation and dephosphorylation. Recently, we found that the D2 fragment of twitchin containing the D2 site (Ser-4316) and flanking immunoglobulin motifs (TWD2-S) formed a heterotrimeric complex with myosin and with actin in the region that interacts with myosin loop 2 (Funabara, D., Hamamoto, C., Yamamoto, K., Inoue, A., Ueda, M., Osawa, R., Kanoh, S., Hartshorne, D. J., Suzuki, S., and Watabe, S. (2007) J. Exp. Biol. 210, 4399–4410). Here, we show that TWD2-S interacts directly with myosin loop 2 in a phosphorylation-sensitive manner. A synthesized peptide, CAQNKEAETTGHKKRKSSA, based on the myosin loop 2 sequence (loop 2 peptide), competitively inhibited the formation of the trimeric complex. Isothermal titration calorimetry showed that TWD2-S binds to the loop 2 peptide with a $K_{d}$ of (2.44 ± 0.09) × 10$^{-6}$ M$^{-1}$ with two binding sites. The twitchin-binding peptide of actin, AGFAGDDAP, which also inhibited formation of the trimeric complex, bound to TWD2-S with a $K_{d}$ of (5.83 ± 0.05) × 10$^{-6}$ M$^{-1}$ with two binding sites. The affinity of TWD2-S to actin and myosin was slightly decreased with an increase of pH, but this effect could not account for the marked pH dependence of catch in permeabilized fibers. The complex formation also showed a moderate Ca$^{2+}$ sensitivity in that in the presence of Ca$^{2+}$ complex formation was reduced.

Molluscan smooth muscles, such as mussel anterior byssus retractor muscle (ABRM) and adductor muscle, exhibit a low energy cost phase of tension maintenance termed catch. Catch muscle develops active tension following an increase of the intracellular [Ca$^{2+}$] induced by secretion of acetylcholine. Myosin is activated by direct binding of Ca$^{2+}$ to the regulatory myosin light chain and initiates a relative sliding between thick and thin filaments (1). After a decrease of intracellular [Ca$^{2+}$] to resting levels, the catch state is formed where tension is maintained over long periods of time with little energy consumption.

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2 The abbreviations used are: ABRM, anterior byssus retractor muscle; ITC, isothermal titration calorimetry; MOPS, 3-(N-morpholino)propanesulfonic acid.

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EXPERIMENTAL PROCEDURES

Protein Preparations—Live specimens of the mussel Mytilus galloprovincialis were used. Mytilus ABRM was dissected from shells, frozen immediately in liquid nitrogen, and stored at −80 °C until use. ABRM myosin was purified as previously reported (11). F-actin was prepared from acetone powder of chicken fast skeletal muscle (13). Protein concentrations were measured by the Bradford method (Bio-Rad).

Peptide Synthesis—The myosin loop 2 peptide of ABRM myosin heavy chain (residues 390–408, CAQNKAEATTGTHKKRKSST) was synthesized (AJ249991) (14) with an additional Myo-2, and 5:1. The reaction mixture thus prepared was kept on ice for 15 min and subjected to low speed centrifugation (5,000 rpm). Injection parameters were: volume, 10 μl; stirring speed, 300 rpm. Titration was performed at 25 °C by injecting 10 μl of 400 μM synthetic peptide (actin subdomain I or myosin loop 2 peptide) into the ITC cell containing 1.5 ml of 10 μM TWD2-S or S-TWD2-S. All samples were dissolved in 30 mM potassium phosphate buffer, pH 7.0, 4 mM MgCl2, and 1 mM ATP. No EGTA was added to the solution as it disturbed ITC measurements. The data thus obtained were fitted according to the 1:1 model. Displacement of TWD2-S by Ca2⁺ was determined by the 1:1 model

RESULTS

Competitive Cosedimentation Assays—Unphosphorylated TWD2-S was used because the phosphorylated TWD2-S does not form the trimeric complex (11). As shown in Fig. 1A, the pellet of the control sample contained myosin, actin, and TWD2-S, i.e. the trimeric complex. In the presence of low levels of the loop 2 peptide, the complex is dissociated, and TWD2-S is found with both myosin (pellet) and actin (supernatant). At the 1:1 ratio (loop 2 peptide/TWD2-S), the concentration of the loop 2 peptide is about 100 greater than the concentration of myosin heads (−84 μM). As the level of loop 2 peptide is increased, more TWD2-S is found in the supernatant, and at the 1:1 ratio is found only in the supernatant. 

The effects of Ca2⁺ on formation of the trimeric complex were monitored by carrying out the cosedimentation assays as above but with 1 mM CaCl2 and 1 μg/ml TWD2-S. Proteins were visualized by silver staining. The concentration of protein in gels was determined by the computer software Image J.

Isothermal Titration Calorimetry—ITC was performed using a VP-ITC calorimeter (MicroCal LLC). Experimental parameters were: total injections, 25 times; cell temperature, 25 °C; reference power, 10 mCal/s; initial delay, 600 s; syringe concentration, 400 μM; cell concentration, 10 μM; stirring speed, 300 rpm. Injection parameters were: volume, 10 μl; duration, 20 s; spacing, 300 s; filter period, 2 s. The effects of Ca2⁺ on formation of the trimeric complex were monitored by carrying out the cosedimentation assays as above but with 1 mM CaCl2 and 1 μg/ml TWD2-S. Proteins were visualized by silver staining. The concentration of protein in gels was determined by the computer software Image J.
on contractions induced at higher \([\text{Ca}^{2+}]\) (6). Thus, it is possible that the formation of the trimeric complex is \(\text{Ca}^{2+}\)-dependent. To address this point, the cosedimentation assays also were carried out in the presence of both ATP and \(\text{Ca}^{2+}\). In the absence of ATP and the presence of \(\text{Ca}^{2+}\) (rigor conditions), actin was cosedimented with myosin without TWD2-S (data not shown). In the absence of \(\text{Ca}^{2+}\) and the presence of TWD2-S (molar ratio to myosin of 1) part of the actin was cosedimented with myosin (Fig. 2A). However, in the presence of \(\text{Ca}^{2+}\), the amount of actin in the pellet was decreased and was calculated to be 46 ± 12% of that cosedimented in the absence of \(\text{Ca}^{2+}\) \((n = 3)\) \((p < 0.05)\). The binding of TWD2-S to the actin and loop 2 peptides in the presence of \(\text{Ca}^{2+}\) slightly decreased as revealed by the solid phase binding assay, supporting the data of the cosedimentation assay (Fig. 2B). Thus, the presence of \(\text{Ca}^{2+}\) partly inhibited the formation of the complex. Part of this effect could be due to a reduced affinity of TWD2-S to myosin and actin in the presence of \(\text{Ca}^{2+}\) and a resultant decrease in formation of the trimeric complex (TWD2-S was detected in the supernatant fraction in the presence of \(\text{Ca}^{2+}\); see arrowhead in Fig. 2A).

**Isothermal Titration Calorimetry**—TWD2-S was titrated by ITC with the loop 2 peptide as shown in Fig. 3A, providing the stoichiometry \((N)\), binding constant \((K_u)\), binding enthalpy \((\Delta H)\), and binding entropy \((\Delta S)\) in the endothermic reaction. The \(N\) value of 1.90 ± 0.02 obtained in a solution containing TWD2-S and the loop 2 peptide indicated that the TWD2-S fragment contained two binding sites for the loop 2 peptide with \(K_u = (2.44 ± 0.09) \times 10^5 \text{ M}^{-1}\). On the other hand, no signal was detected in the reaction mixture containing S-TWD2-S and the loop 2 peptide, indicating that interaction between TWD2-S and the loop 2 peptide is lost on phosphorylation of the D2 site in the twitchin molecule. The same experiment was performed using the actin peptide, which was shown previously to bind to TWD2-S (11). The thermogram and binding isotherm in the titration of the actin peptide with TWD2-S are shown in Fig. 3B. The \(N\) and \(K_u\) values for the binding of the actin peptide to TWD2-S were 1.83 ± 0.10 and \((5.83 ± 0.05) \times 10^4 \text{ M}^{-1}\), respec-
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![Diagram](image)

**FIGURE 2. Cosedimentation assay in the presence of Ca**${}^{2+}$**.** A, SDS-PAGE patterns of the supernatants and pellets obtained after centrifugation. In the presence of Ca$^{2+}$, the amount of actin cosedimented with myosin decreased compared with that in the absence of Ca$^{2+}$. A part of TWD2-S was not cosedimented with myosin in the presence of Ca$^{2+}$. The arrowhead indicates the TWD2-S that was not cosedimented with myosin. B, binding of TWD2-S to the loop 2 and actin peptides in the presence of Ca$^{2+}$. Filled and open columns represent the absorbance obtained in solid phase binding assays for the loop 2 and actin peptides, respectively, using TWD2-S as a probe. Data are shown with mean values ± S.D. (n = 3) and significant differences at the level of *p* < 0.05 (*). Refer to Fig. 1 for the abbreviations used.

EFFECTS OF pH ON BINDING OF TWD2-S TO MYOSIN, THE LOOP 2 PEPTIDE, AND ACTIN—It was reported that a change of pH affects catch contraction, and higher pH (in the range of 7.2–7.7 in various studies) (see Ref. 15) in skinned muscle fibers caused inactivation and does not bind to actin (Fig. 2A), indicating that TWD2-S contained two binding sites for the actin peptide. Thiophosphorylation of TWD2-S diminished the binding of S-TWD2-S to the actin peptide as in the case with S-TWD2-S and the myosin loop 2 peptide (Fig. 3, A and B, lower panels). Data from ITC together with the above competitive cosedimentation assay clearly demonstrate that both myosin loop 2 and the actin subdomain I regions are involved in the formation of the trimeric complex of myosin, actin, and twitchin.

**DISCUSSION**

In the present study, it is shown that the myosin loop 2 region is involved in the formation of the trimeric complex of myosin, actin, and twitchin. The twitchin-binding region of actin, located in subdomain I, also is known as the myosin loop 2-binding region. Thus, it is reasonable to assume that the formation of the trimeric complex involves interactions of twitchin with actin subdomain I and with the myosin head loop 2 region. The colocalization of twitchin in the region of the D2 site with myosin and actin is supported by previous electron microscope observations of isolated thick filaments (11). The electrostatic interaction of myosin loop 2 with actin is implicated in the first step of the ATP-driven movement (16). An increase in [Ca$^{2+}$], initiates the active state by Ca$^{2+}$ binding to myosin and also leads to dephosphorylation of twitchin by calcineurin (17). As suggested previously (11), the formation of the high force state is thought to displace the twitchin interactions with myosin and actin and thus prevent formation of catch. This is consistent with observations made with reconstituted fibers (18). Thus, during an active contraction, the competition between the actin-myosin interaction sites and the sites on actin subdomain I and on myosin loop 2 for twitchin would be lessened by formation of the high force state. From the present study, it can be added that Ca$^{2+}$ also decreased the binding of actin to myosin in the presence of TWD2-S, probably reflecting a reduction in binding of the twitchin peptide to myosin and actin, as shown. This would reduce the possibility for formation of catch in the presence of Ca$^{2+}$, facilitating the interaction between actin and myosin filaments.

It is interesting that ITC experiments indicated two binding sites on the twitchin fragment for both the actin peptide and the loop 2 peptide. How these four sites are regulated by phosphorylation of the D2 site is not known, and the relationship, if any, between the loop 2 sites for actin and twitchin and the actin sites for loop 2 and for twitchin requires clarification. The data from the cosedimentation studies suggest that the link between twitchin (TWD2-S) and myosin is more sensitive to competition by the loop 2 peptide. At 1:10 loop 2 peptide/TWD2-S, the interaction between actin and myosin is lost.

Under these conditions, the concentration of loop 2 peptide is about 10-fold higher than the myosin head concentration. These findings indicate that there is no direct interaction between myosin and actin when the two proteins form the trimeric complex with twitchin. Under catch condition, myosin is inactivated and does not bind to actin (Fig. 2A), indicating that the loop 2 region does not bind to actin. In the cosedimentation assay, loop 2 peptide would have competed with the loop 2 region in myosin for binding to TWD2-S. It is noted that the cosedimentation assay was performed at cold temperature to...
avoid any possible denaturation of myosin and actin, whereas the ITC experiments were carried out at 25 °C to facilitate the quick reaction required for the experiments between the peptides. However, proteins or peptides are well known to change their structures dramatically depending on environmental temperatures. Therefore, such possible structural differences must be carefully considered to interpret the data obtained in the two series of the experiments in the present study.

Galler and co-workers (15) reported that structures within the myofilament that are sensitive to pH changes are responsible for catch. Our study on the pH dependence of the interactions of TWD2-S showed that its binding to myosin and actin was reduced on increasing pH, but its interaction with the loop 2 peptide was not affected. With the fiber studies, the pH was critical for formation of catch, and catch was not observed at pH values around 7.4 (15). The binding of TWD2-S although slightly reduced at these pH values still occurred. Thus, the marked pH dependence seen with fibers cannot be attributed entirely to a pH dependence of the interactions involved in formation of the trimeric complex. These results are consistent with previous observations (11) where the trimeric complex was formed at pH 7.4. However, the salt concentration adopted in the present study was 200 mM KCl, which is somewhat higher than those used elsewhere for fibers. Therefore, experiments in different salt concentrations are required for a more correct interpretation of the results obtained in the present study.

A controversial and long standing issue is the identity of the physical interaction(s) responsible for maintaining tension in catch with low energy consumption. Recent studies have proposed that the myosin cross-bridge-actin interaction is not involved (10, 19–21). Our results are consistent with this idea, and we propose that interaction between thick and thin filaments is due to the formation of a complex between actin and myosin that is cross-linked by twitchin, specifically in the region around the D2 site. The myosin site for interaction with twitchin is in the myosin head (the loop 2 region) but distinct

![Isothermal titration calorimetry for the interaction of TWD2-S or thiophosphorylated TWD2-S with the myosin loop 2 and actin peptides.](image-url)

**FIGURE 3.** Isothermal titration calorimetry for the interaction of TWD2-S or thiophosphorylated TWD2-S with the myosin loop 2 and actin peptides. A, the titration of TWD2-S with the loop 2 peptide. B, the titration of TWD2-S with the actin peptide. Titration was performed in 30 mM potassium phosphate buffer, pH 7.0, containing 4 mM MgCl₂ and 1 mM ATP at 25 °C. Upper and lower panels show thermograms and binding isotherms, respectively. The results from the titration of S-TWD2-S with the loop 2 and actin peptides are represented in the lower panels (open circles) together with those of TWD2-S (closed circles). Stoichiometry (N), binding constant (Kₐ), binding enthalpy (ΔH), and binding entropy (ΔS) were calculated and are presented in insets in the lower panels of A and B.
from the cross-bridge. The trimeric complex, however, may be sensitive to cycling cross-bridge–actin interactions because it is proposed that the complex is not formed or is displaced in the high force state.

Further studies are necessary to define and localize the various sites involved in formation of the trimeric complex. It is also important to determine whether or not the trimeric complex is formed by the reaction of twitchin with myosin and actin, cooperatively.

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FIGURE 4. The pH dependence of binding of TWD2-S to myosin, its loop 2 peptide, and actin. Black, white, and gray columns represent the absorbance obtained in solid phase binding assays for myosin, the loop 2 peptide and actin, respectively, using TWD2-S as a probe. Hybridization was carried out in 50 mM MOPS containing 1 mM EGTA and 200 mM KCl adjusted to pH 6.7, 7.2, 7.4, and 7.7 at room temperature. Data are shown with mean values ± S.D. (n = 5). Data for myosin and actin at pH 7.2, 7.4, and 7.7 are significantly different from those at pH 6.7 at levels of p < 0.01 (**) and p < 0.05 (*), respectively.