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Ionic interaction of myosin loop 2 with residues located beyond the N-terminal part of actin probed by chemical cross-linking

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Abstract

To probe ionic contacts of skeletal muscle myosin with negatively charged residues located beyond the N-terminal part of actin, myosin subfragment 1 (S1) and actin split by ECP32 protease (ECP-actin) were cross-linked with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). We have found that unmodified S1 can be cross-linked not only to the N-terminal part, but also to the C-terminal 36 kDa fragment of ECP-actin. Subsequent experiments performed on S1 cleaved by elastase or trypsin indicate that the cross-linking site in S1 is located within loop 2. This site is composed of Lys-636 and Lys-637 and can interact with negatively charged residues of the 36 kDa actin fragment, most probably with Glu-99 and Glu-100. Cross-links are formed both in the absence and presence of MgATP.P_i analog, although the addition of nucleotide decreases the efficiency of the cross-linking reaction.

Keywords: Actin–myosin interaction; Subfragment 1; Chemical cross-linking

1. Introduction

Myosin is a motor protein that when associated with actin transforms the chemical energy of ATP into movement. During the ATPase cycle, the initial weakly bound complex, where the actin–myosin interaction is predominantly ionic, undergoes the multistep transition into the strongly-bound complex, where the contacts are both of hydrophobic and ionic in nature. Identifying specific interactions between actin and myosin is critical for understanding how actin activates myosin ATPase, and for determining how force generation is coupled to the ATPase cycle.

The head part of the myosin molecule, subfragment 1 (S1), contains proteolytically sensitive, flexible loops that play an important role in myosin function. Many chemical cross-linking studies indicate that ionic contacts between F-actin and myosin involve the positively charged loop 2, comprising residues 626–647 and connecting the central 50 kDa and C-terminal 20 kDa segments of the S1 heavy chain [1–5]. In the myosin of skeletal muscle there is also another lysine-rich subsite, located in the 50 kDa heavy chain segment and called the secondary actin-binding loop (residues 567–578) that together with loop 2 contributes to the interaction of a single myosin head with two adjacent actin monomers [2–5].

It is well documented by cross-linking studies using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) that in skeletal muscle ionic interaction between F-actin and myosin involves a short segment of actin, containing 1–4 negatively charged N-terminal residues [6,7]. Data from mutational studies indicate that in addition to the residues located at the N-terminus of actin, other negatively charged residues, 24/25 and 99/100, also contribute to the acto-S1 interaction, affecting actomyosin function. N-terminal residues are involved in both weak and strong binding to myosin, while the 24/25 and 99/100 sites contribute to the weak acto-S1 binding [8–11]. All three sites are located in subdomain 1 of actin [12].

Abbreviations: S1, myosin subfragment 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; IAEDANS, *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; ECP32, proteinase from *E. coli* A2 strain

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Models of the rigor actomyosin complex that have been built using X-ray structures of individual proteins and electron micrographs of F-actin saturated by S1, have provided insight into the intermolecular contacts between actin and myosin. Although surface loops of S1 are not present in the X-ray structure, S1 loop 2 is expected to interact by ionic contacts with segment 1–4 and residues 24/25 of one actin subunit, while 567–578 loop could potentially interact with residues 99/100 of the second actin monomer [13,14]. However, it has been shown that both adjacent actin subunits are cross-linked with EDC to a single myosin head through their N-terminal 1–28 residue segments [7]. Until now, chemical cross-linking studies have failed to confirm a contribution of 99/100 actin site to the acto-S1 interaction.

The aim of this work was to determine S1 site(s) that can form ionic contacts with residues located beyond the N-terminal part of actin. We used EDC to induce cross-linking between actin cleaved with ECP32 protease (ECP-actin) and native or proteolytically split S1. The obtained results show for the first time that in the lysine-rich sequence of loop 2 – Lys-Lys-Gly-Gly-Lys-Lys-Lys (residues 636–642) – a site composed of Lys-636 and Lys-637 can interact with negatively charged residues of actin other than those located at its N-terminal part, most probably with residues Glu-99 and Glu-100. These contacts between myosin loop 2 and F-actin are formed (although with different efficiency) both in the presence and absence of MgADP.P_i analog. The cross-linking of S1 to the above actin site was not observed when the segment of loop 2 containing Lys-636 and Lys-637 residues was removed by proteolysis.

2. Materials and methods

2.1. Materials

The protease ECP32 from *E. coli* A2 strain was kindly provided by Dr. A. Morozova (Institute of Cytology, St. Petersburg, Russia). ATP, ADP, chymotrypsin, trypsin, elastase, soybean trypsin inhibitor, EDC and *N*-iodoacetyl-*N*′-(5-sulfo-1-naphtyl)ethylenediamine (IAEDANS) were obtained from Sigma.

2.2. Proteins

Myosin was prepared from rabbit back muscles, and S1 was obtained by digestion of myosin with chymotrypsin [15].The S1(A2) was separated from S1 (A1) isoform by step elution from a SP Tris–acryl M column [16]. Actin was obtained as described in [17].

2.3. Labeling of proteins

F-actin was labeled at Cys-374 and S1 was labeled at Cys-707 with the fluorescent dye 1,5-IAEDANS according to [18] and [19], respectively.

2.4. Preparation of ECP-actin

After depolymerization, labeled or non-labeled G-actin was cleaved with partially purified ECP32 [20] for 2 h at 25 °C. Precise conditions of the reaction (quantity of the enzyme, time of digestion) were established by examining the extent of actin cleavage with the use of SDS-PAGE. The cleaved actin was used within 10–12 h. Before mixing with S1 for cross-linking, G-actin was transformed into Mg-bound form by 5 min incubation with 0.2 mM EGTA and 0.1 mM $MgCl_2$, and then polymerization was started by the addition of 25 mM NaCl, in the presence of phalloidin added at 1:1 molar ratio to actin.

2.5. Preparation of S1 derivatives

The S1 derivatives were obtained by digestion of S1 with elastase or trypsin at 25 °C, in 25 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.5. The digestion of S1 with elastase was performed at the enzyme to substrate ratio of 1:10 (w/w) for 20 min, then the reaction was stopped by the addition of 1 mM PMSF; in certain experiments the digestion medium also contained 10 mM ATP that was subsequently removed by dialysis. The digestion of S1 with trypsin was performed at the enzyme to substrate ratio of 1:25 (w/w) for 15 min, and stopped by the addition of soybean trypsin inhibitor at 1.5:1 (w/w) ratio to trypsin.

2.6. Preparation of S1-nucleotide-aluminium fluoride complexes

The complexes of S1 or S1 derivatives with MgADP.AlF_4 were obtained in a medium containing 25 mM NaCl, 10 mM HEPES, pH 7.5, by incubation of S1 (25 mμ) for 10 min at 25 °C in 2 mM $MgCl_2$, 2 mM ADP, 9 mM NaF, 2 mM $AlCl_3$.

2.7. Cross-linking of S1 to F-actin

For the cross-linking reaction, S1, S1 derivatives or S1-MgADP.AlF_4 complexes (13 mμ) were mixed with proteolytically modified or unmodified actin (28 mμ), kept for 1 h at 25 °C in 25 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.5, and then incubated with 20 mM EDC for 10 or 20 min at 25 °C. The reaction was terminated by addition of 70 mM 2-mercaptoethanol.

2.8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The cross-linking products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to [21].

2.9. Amino acid sequence analysis

The 20 kDa peptide obtained by digestion of S1 with elastase and separated by SDS-PAGE was electrophoretically transferred to a polyvinylidene fluoride (Immobilon P^{SQ}) transfer membrane [22]. The blotted band was sequenced on an ABI Procise Model 492 using the pulsed liquid protocol and PTH-amino acids identified by on-line analysis based on a 10 pmol PTH standard.

3. Results

Actin is cleaved by ECP32 protease between Gly-42 and Val-43 into two parts that remain associated: a relatively small N-terminal peptide and a larger C-terminal 36 kDa fragment [20]. Although treatment with ECP32 protease reduces acto-S1 ATPase activity, this inhibitory effect can be largely reversed by stabilization of intersubunit contacts in F-actin with phalloidin [23]. For the reason stated above, in our experiments ECP-actin was polymerized in the presence of this stabilizing reagent. Cross-linking between ECP-actin and S1 was induced by treatment of the proteins with EDC. Actin in the mixture was present in two fold molar excess over S1. To avoid problems caused by the presence of the A1 light chain that can obscure the reaction results, experiments were performed on S1(A2) isoform. Fig. 1 compares electrophoretic patterns of S1 cross-linked either to cleaved or non-cleaved actin. ECP-actin is represented by its C-terminal 36-kDa fragment; the N-terminal peptide migrates too fast to be retained in the gels. Cross-linking of ECP-actin to S1 generated two main products of molecular masses of about 100 kDa and 165 kDa. Since the first product was not found on the electrophoretic pattern when S1 was cross-linked to intact actin, it must be composed of the S1 heavy chain and the N-terminal fragment of ECP-actin. The relatively less intense