ISSN 0006-2979, Biochemistry (Moscow), 2018, Vol. 83, No. 8, pp. 944-960. © Pleiades Publishing, Ltd., 2018.
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REVIEW

Essential Light Chains of Myosin and Their Role in Functioning of the Myosin Motor

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Received February 27, 2018
Revision received April 13, 2018

Abstract—This review summarizes current data on the structure and functions of myosin essential light chains (ELCs) and on their role in functioning of the myosin head as a molecular motor. The data on structural and functional features of the *N*-terminal extension of myosin ELC from skeletal and cardiac muscles are analyzed; the role of this extension in the ATP-dependent interaction of myosin heads with actin in the molecular mechanism of muscle contraction is discussed. The data on possible interactions of the ELC *N*-terminal extension with the myosin head motor domain in the myosin ATPase cycle are presented, including the results of the authors' studies that are in favor of such interactions.

DOI: 10.1134/S0006297918080060

Keywords: myosin, myosin essential light chains, actin, molecular mechanism of muscle contraction

Cyclic interactions between myosin heads and actin filaments accompanied by ATP hydrolysis underlie molecular mechanisms that provide a broad range of biological motility types – from intracellular transport to muscle contraction. All muscle myosins in a large myosin superfamily (~35 different classes) belong to class II. Class II myosins consist of two heavy and four light chains. The *N*-terminal parts of heavy chains form globular heads, each containing the ATPase catalytic site and actin-binding sites. Myosin head by itself is a molecular motor capable of performing independent motor functions [1, 2]. Isolated myosin head termed myosin subfragment 1 (S1) consists of two major structural domains known as the motor (or catalytic) domain and the regulatory domain. The motor domain is responsible for ATP hydrolysis and actin binding, whereas the regulatory domain is represented by a long α-helix stabilized by non-covalent interactions with two light chains: the essential light chain (ELC) and the regulatory light chain (RLC) [3]. According to modern concepts, the functioning of the myosin head as a molecular motor includes regulatory domain rotation relatively to the motor domain, in which regulatory domain acts as a lever arm that potentiates conformational changes occurring in the motor domain during the ATPase cycle [2, 4-6]. The greatest conformational changes are associated with the formation of intermediates of the ATP hydrolysis by myosin – M*-ATP and M**-ADP-P_i. However, these intermediates are very short-lived, which makes it impossible to study in detail the myosin head structure in such complexes. For this purpose, stable analogs of the reaction intermediates have been successfully used, such as the myosin head (S1) triple complexes with ADP and P_i analogs, including ortovanadate (V_i), beryllium fluoride (BeF_x), and aluminum fluoride (AlF_4^-) anions [2, 7, 8]. It was established that the S1-ADP-BeF_x complex mimics the S1*-ATP intermediate, whereas the S1-ADP-V_i and S1-ADP-AlF_4^- complexes mimic the S1**-ADP-P_i intermediate [9, 10]. These stable analogs have been success-

Abbreviations: A1 and A2, isoforms of skeletal muscle myosin essential (alkali) light chains; AlF_4^-, aluminum fluoride anion; BeF_x, beryllium fluoride anion; DLS, dynamic light scattering; DSC, differential scanning calorimetry; ELC, myosin essential light chain; ELCa, atrial ELC; ELCv, ventricular ELC; FHC, familial hypertrophic cardiomyopathy; FRET, Forster resonance energy transfer; *MM*, molecular mass; RLC, myosin regulatory light chain; S1, myosin subfragment 1; S1(A1) and S1(A2), myosin subfragment 1 isoforms containing alkali light chains A1 and A2, respectively.

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fully used in crystallographic studies of the myosin head [6, 11]. In 1998, the atomic structure of the smooth muscle S1 in the S1-ADP-BeF_x and S1-ADP-AlF_4^- complexes was determined. It was shown that rotation of the regulatory domain relatively to the motor domain during the ATPase cycle could be accompanied by a rather strong interaction between the S1 motor domain and the *C*-terminal part of the ELC associated with the regulatory domain [11].

In this review we discuss the structural features of different isoforms of myosin ELCs from skeletal and cardiac muscles and the role of ELCs in the motor function of the myosin head. But first we would like to explain why these light chains are called essential.

The commonly accepted term *essential light chains* (ELCs) is associated with the earlier idea that they are absolutely necessary for the manifestation of myosin's major properties – ATPase activity and actin binding. ELCs are also called *alkali* light chains, because for a long time they could be separated from the heavy chains only under rather harsh conditions (in particular, high pH values) that nearly always led to the irreversible loss of the major myosin activities. However, in the early 1980s, two groups of researchers independently and nearly synchronously succeeded in isolating ELC-free S1 heavy chains that retained their ATPase activity and ability to bind actin [12, 13]. Although the term *essential* is still used by some authors, it is often put in inverted comas, since it has become clear that this term does not reflect the actual role of ELCs.

ELC ISOFORMS AND THEIR STRUCTURAL FEATURES

Muscle express two major ELCs isoforms – the long and the short ELCs. Smooth muscles, mammalian non-muscular cells, and muscles of invertebrates contain only short ELC isoforms (up to 150 a.a.). Long ELC isoforms (188 to 204 a.a.) are typical for skeletal and cardiac muscles of animals and humans. Both long and short ELC isoforms are expressed simultaneously only in fast skeletal muscles.

ELC isoforms in fast skeletal muscles. Myosin from fast skeletal muscles of vertebrates contains the A1 (long) and A2 (short) ELC isoforms. The names come from the English terms *Alkali 1* and *Alkali 2* and are traditionally used in the literature. The molecular masses (*MM*) of A1 and A2 from rabbit fast skeletal muscles are 20.7 and 16.5 kDa, respectively [14]. Both A1 and A2 are encoded by the *MYL1* gene (HUGO Gene Nomination Committee). Generation of two ELC isoforms is a result of gene transcription from different promoters and alternative splicing of the formed mRNAs [15, 16]. A1 and A2 from human skeletal fast muscles consist of 194 and 150 a.a., respectively. The two isoforms have identical *C*-terminal sequence (141 a.a.) that contains a single Cys residue (Cys181 in A1 from human muscles; Cys137 in A1 from rabbit muscles; Cys177 in A2 from human muscles; Cys136 in A2 from rabbit muscles). The *N*-terminal fragment of A2 (8 to 9 residues) differs from the corresponding fragment of A1 by 6 and 5 amino acid residues in human and rabbit myosins, respectively. However, the main difference between A1 and A2 is the presence in A1 of an additional *N*-terminal sequence consisting of 44 and 41 a.a. in human and rabbit myosins, respectively. This sequence contains a fragment enriched with Ala-Pro repeats and two pairs of positively charged Lys residues at the *N*-terminus [14, 17]. The differences between the A1 and A2 sequences are shown in Fig. 1.

It is this *N*-terminal extension in A1 that determines the differences in the properties of A1 and A2 isoforms. Each of the two heads in the myosin molecule from fast skeletal muscles contains only one ELC isoform (either

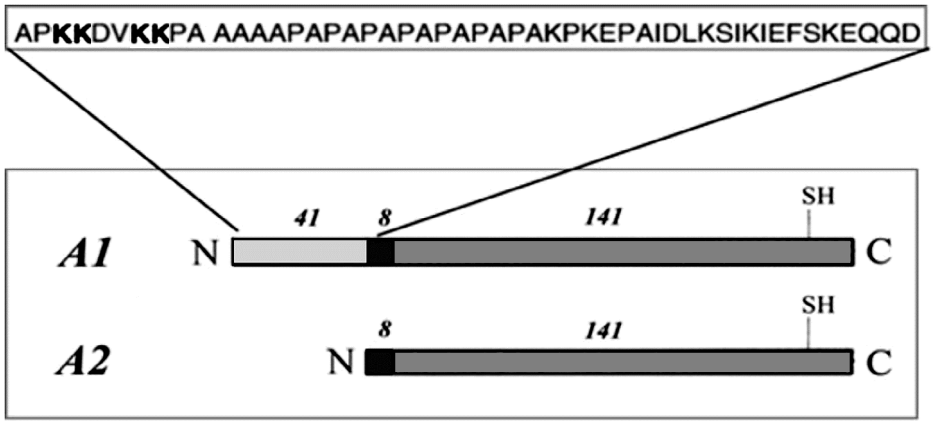

Fig. 1. Differences in the primary structures of myosin ELC isoforms A1 and A2 from fast skeletal muscles of vertebrates. The full sequence is shown only for the *N*-terminal extension in A1 (absent in A2). The *C*-terminal fragments (141 a.a.) of A1 and A2 are identical. See the text for details.

A1 or A2), so that myosin exists either as a homodimer with identical ELCs in both heads or a heterodimer containing different ELC isoforms. The method for S1 separation into A1- and A2-containing fractions by ion-exchange chromatography was developed in 1970s [18] and has been significantly improved since then [19]. The properties of S1(A1) and S1(A2) preparations are frequently studied in comparison to reveal the functional role of the *N*-terminal extension in A1. The results of these studies will be discussed below in this review.

When analyzing the amino acid sequence of the *N*-terminal extension in A1 (Fig. 1), it should be noted that an important role in the structure of this sequence is played by positively charged Lys residues at the *N*-terminus, as well as by the downstream region containing Ala-Pro repeats. According to the crystallography data, a peptide consisting of four α-aminoisobutyric acid-Pro repeats (analog of Ala-Pro repeats in the A1 protein structure) has a very rigid conformation [20]. The authors of [21] used mathematical approaches to analyze the conformation of the fragment with Ala-Pro repeats in the *N*-terminal extension of A1 (a.a. 14-27) and found that it represents a rod-like structure with a limited flexibility ~4 nm in length, so that the entire length of the *N*-terminal extension is 8-9 nm [21, 22]. This structural feature of the *N*-terminal extension of A1 can play an important functional role in the interaction of S1(A1) with actin (see below).

Cardiac ELC isoforms. Heart atria and ventricles express different ELC isoforms – ELCa (atrial ELC) and ELCv (ventricular ELC), encoded by the *MYL4* and *MYL3* genes, respectively [23, 24]. *MYL3* also encodes ELC in slow skeletal muscles. The structures of ELCa and ELCv resemble that of the A1 long isoform from fast skeletal muscles, although with some differences. ELCa consists of 197 a.a. and contains two Cys residues in its central and *C*-terminal fragments; ELCv consists of 195 a.a. and contains three Cys residues (unlike A1 that contains a single Cys residue in the *C*-terminal fragment). ELCa and ELCv also contain five and six positively charged Lys residues, respectively, within the first 20 a.a. of their *N*-terminal sequences [17] (A1 has only four Lys residues in this region).

During embryogenesis, ELCa is expressed in atria and ventricles [25], as well as in the skeletal muscles, while in adult organisms, ELCa is synthesized only in the atria. ELCa can also be expressed in the ventricles of patients with different cardiomyopathies. Despite a high degree of homology between the amino acid sequences of ELCa and ELCv [26], the presence of ELCa in the ventricles improves the overall cardiac activity [27, 28]. Thus, studies on muscle fibers isolated from the ventricles during heart transplantation in patients with different cardiomyopathies showed that partial replacement of ELCv with ELCa in the ventrical myosin increased the maximal rate of fiber contraction and elevated contraction sensitivity to calcium. Partial replacement of ELCv with ELCa in heart ventricles is now believed to be a compensatory response in heart failure [27, 28]. Thus, it has been shown that ELCa expression in ventricles of transgenic mice and rats improved the rate and general efficiency of ventricle contractions in these animals [29]. The observed increase in the heart activity was not accompanied by the cardiac hypertrophy development. Apparently, in some cases ELCa expression in the ventricles can be beneficial and results in strengthening the cardiac function [30]. Interestingly, regular physical activity increased the level of ELCa in the ventricles and improved heart contractility in rats [31]. The ELCa-mediated strengthening of cardiac muscle was confirmed at the level of individual myosin molecules. Thus, partial replacement of 12, 24, and 42% ELCv in ventricles with the recombinant ELCa increased the ATPase activity of actomyosin by 18, 26, and 36%, respectively [32].

It was found that substitution of ELCs in the S1 preparation from rabbit skeletal muscles with the recombinant human ELCa produced almost no effect on the actin-stimulated ATPase activity of S1(A1); however, removal of 45 *N*-terminal residues from ELCa brought this activity to the level typical for S1(A2). The activity of the S1 preparation containing recombinant ELCa lacking 11 *N*-terminal residues was intermediate between those of S1(A1) and S1(A2), although its actin-stimulated ATPase activity was close to the activity of S1(A2) [33]. This indicates that the *N*-terminal fragment plays an important role played in the functioning of cardiac ELC and is similar to the *N*-terminal extension of A1 from fast skeletal muscles (Fig. 1). It cannot be excluded that the differences in the properties of ELCv and ELCa are caused by the difference in the the *N*-terminal extensions of these cardiac ELC isoforms [17]. The functions of the *N*-terminal extensions in the ELCs from skeletal and cardiac muscles will be discussed in a special section of this review. However, at first, we shall describe the functions of the ELC *C*-terminal fragment that is similar in all ELC isoforms – both long (A1-like) and short (A2-like) ones.

FUNCTIONS OF THE ELC *C*-TERMINAL PART

The role of the ELC *C*-terminal part in stabilization of the regulatory domain of the myosin head. The major function of all ELC isoforms, both long and short ones, is stabilization of the myosin heavy chain α-helix in the regulatory domain of the myosin head. ELCs bind to the myosin head heavy chain in the region of the IQ motifs (Fig. 2) so that the two halves of the ELC molecule are located at the sides of the α-helix. Such location of the ELC in the myosin head is characteristic of all ELC isoforms and was found in crystal structures of S1 from chicken skeletal [3] and smooth [11] muscles, as well as in S1 and isolated S1 regulatory domain from scallop adduc-

tor muscles [6, 34]. No data on the structure of free (not bound to myosin heavy chain) ELCs are available so far. Most probably, free ELCs exist in the unfolded state.

Early studies of the myosin structure had shown that isolated ELC-free heavy chains of S1 are very unstable and rapidly lose their ability to hydrolyze ATP and interact with actin [35, 36], while re-association with ELCs partially restores this ability [36]. Based on these data, it was concluded that ELCs stabilize the myosin head structure in the regions of the ATPase catalytic site and actin-binding sites. Later, an important role of ELCs in the myosin head functioning in skeletal muscle has been confirmed in *in vitro* motility assays. It was shown that removal of the two light chains (both ELCs and RLCs) from chicken skeletal myosin drastically decreased the sliding velocity of actin filaments without a significant loss in the ATPase activity of actomyosin; re-association of myosin heavy chains with ELCs partially restored the filament sliding velocity [37, 38]. In [38], the authors used glass microneedle technique to measure the force developed by myosin heads during their interaction with a single actin filament under isometric conditions. It was shown that selective removal of ELCs from myosin reduced the isometric force by more than 50%, whereas the RLC removal had very little effect on the isometric force [38]. The results of these experiments showed that ELCs play a critical role in motor functions of the myosin head by stabilizing its regulatory domain with which they interact through their *C*-terminal parts.

The importance of the ELC *C*-terminal part in cardiac myosin is evidenced by the correlation between the presence of certain mutations in this ELC part and the development of such severe hereditary disease as cardiomyopathy [17].

Cardiomyopathic mutations in the C-terminal part of cardiac ELC isoforms. Clinical studies have shown that impairments in the primary structure of human ELCv caused by mutations in the *MYL3* gene could be associated with Familial Hypertrophic Cardiomyopathies (FHCs). FHCs are dominant autosomal diseases characterized by hypertrophy of the left ventricle wall. Very frequently, FHCs cause instant death not preceding by symptoms or warnings [39]. The clinical phenotype of FHC is very broad – from full absence of cardiovascular symptoms to the pronounced dyspnea and chest pain. FHCs are caused by point mutations or deletions in genes encoding major sarcomeric proteins, such as cardiac myosin heavy chains, titin, actin, α-tropomyosin, C-protein, RLC, ELC, and troponins T, I, and C. By now, seven FHC-associated amino acid substitutions in human ELCv are known that are caused by mutations in the *MYL3* gene: E56G, A57G, E143K, M149V, R154H, M173V, and E177G [17, 40-45]. It should be noted that all these substitutions are localized to the ELCv region interacting with the heavy chain α-helix in the myosin head regulatory domain. No mutations associated with FHC have been detected in the *N*-terminal part of ELCv (50 residues corresponding to the *N*-terminal extension in A1) that includes functionally important Ala-Pro repeats (residues 16-27) and positively charged Lys residues at the *N*-terminus. All mutations in the *MYL3* gene leading to the FHC development are located in conserved regions of the ELCv *C*-terminal part, except the two mutations (E56G and A57G) that are situated close to the *N*-terminal part of ELCv.

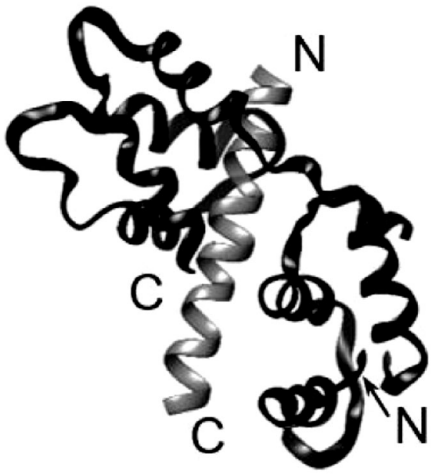

Fig. 2. Interaction of ELC (black) with the myosin heavy chain α-helix (grey) in the myosin head regulatory domain (PDB entry 2MYS). See the text for details.

The E143K mutation in *MYL3* gene might cause death at birth or at a young age. It was shown recently [46] that transgenic mice expressing human ELCv with the E143K mutation develop interstitial fibrosis and systolic dysfunctions and display an increased force of the muscle fiber contractions. At the molecular level, the E143K mutation increased the binding affinity of myosin to actin and elevated the ATPase activity of actomyosin.

Many studies have been focused on the functional effects of the A57G mutation that was first discovered in two Korean families with FHC and a Japanese patient with the classic asymmetric septal hypertrophy [41]. The effects of this mutation have been comprehensively studied in the cardiac muscle of A57G transgenic mice [47, 48], where it leads to the development of fibrosis and impaired cardiac muscle morphology. Studying the contractility of muscle fibers isolated from the hearts of A57G transgenic mice showed that the A57G mutation decreases the maximal force developed by the fibers but enhances the contraction sensitivity to calcium [47].

The impact of cardiomyopathic mutations in the *MYL3* gene on the cardiac muscle functioning and contractility of cardiac muscle fibers can be explained by destabilization of the myosin head regulatory domain via decreasing the ELCv affinity for its binding site (IQ motif) in the heavy chain α-helix. A series of elegant

experiments using plasmon resonance demonstrated that M149V, E143K, A57G, and R154H mutations significantly (2-3-fold) decreased the ELCv affinity for the recombinant fragment of the cardiac myosin heavy chain containing this IQ motif. The most pronounced effect was observed for the E56G mutation that drastically decreased the ELCv affinity for the IQ motif (the dissociation constant increased more than 30 times) [49]. Moreover, the E56G mutation in ELCv decreased the rigidity of the entire myosin molecule, as well as the velocity of myosin-catalyzed sliding of actin filaments in the *in vitro* motility assay [50].

Interesting data have been obtained recently in the studies using time-resolved fluorescence energy transfer (TR-FRET). TR-FRET makes it possible to rapidly measure the distances between the myosin head and actin directly in the course of the actomyosin ATPase cycle, when the myosin head affinity for actin changes from low to high and *vice versa*. Moreover, this method also allows to determine the relative number of S1 molecules weakly and strongly bound to actin in the presence of ATP. It was found that the E56G mutation markedly (by 33%) increases the population of S1 molecules strongly bound to actin. The authors suggested that this shift toward strong binding could lead to the development of increased heart contractility in patients with the cardiomyopathic E56G mutation in *MYL3* gene coding for myosin ELCv [51].

It should be noted that the cardiomyopathic mutations E56G and A57G are located near the ELCv *N*-terminal extension (residues 1-45) that plays an important role in the myosin head function in skeletal and cardiac muscles (see below). It cannot be excluded that disorders in the contractile functions of muscle fibers resulting from the E56G and A57G mutations can be caused not by destabilization of the myosin head regulatory domain only but also by the impact of these mutations on functioning of the *N*-terminal extension of ELCv.

Cardiomyopathic mutations in ELCv have been attracting a lot of interest recently. Although some of them (e.g., A57G and E56G) had already been shown to influence myosin properties and contractility of muscle fibers, the impact of other ELCv mutations (e.g., M149V and E177G) and especially, the molecular mechanisms of their action, remain unknown.

The role of the ELC *C*-terminal part in the interactions between the motor and regulatory domains of the myosin head in the ATPase cycle. The data on a presumed role of ELC, namely, its *C*-terminal part associated with the myosin head regulatory domain, in the interactions between the myosin head motor and regulatory domains supposedly occurring during the ATPase cycle are scarce and frequently contradictory.

According to the modern concept, the function of the myosin head as a molecular motor involves rotation at ~60° of the regulatory domain relatively to the motor domain. The regulatory domain acts as a lever arm, whose length determines the distance of the myosin head translocation along the actin filament. Elongation/shortening of the regulatory domain by the introduction/removal of the ELC-binding sites increases or decreases, respectively, the efficiency of actin filament translocation by immobilized S1 molecules in the *in vitro* motility assay [2].

Detailed analysis of the crystal structure of the chicken skeletal muscle S1 [3] revealed that, in addition to the ELC binding to the long α-helix of the head regulatory domain, heavy chains of the S1 motor domain interact with the ELC *C*-terminal part associated with the S1 regulatory domain [52]. It was established that residues 90-96 in the ELC *C*-terminal part are located in immediate vicinity to residues 720-730 of the motor domain heavy chain; residues 103-115 of the ELC are located close to the *N*-terminal part of the S1 heavy chain (residues 21-31). It was suggested that these contacts between the S1 motor and regulatory domains, more accurately, between certain regions of the motor domain and the ELC *C*-terminal part in the regulatory domain could play an important or even critical role in the molecular mechanisms of muscle contraction [52].

A little later, in 1998, a group of American scientists solved the structures of smooth muscle S1 containing a fragment of the regulatory domain and ELC in the triple S1-ADP-BeF_x and S1-ADP-AlF_4^- complexes mimicking the major intermediates of the myosin ATPase reaction S1*-ATP and S1**-ADP-P_i, respectively [11]. It was found that the ELC *C*-terminal part forms tight contacts with the motor domain loop (the so-called 25/50 kDa loop) near the nucleotide-binding site. This interaction is fundamentally different from the contacts between the motor and regulatory domains of S1 detected earlier in the structure of the nucleotide-free skeletal muscle S1 [3, 52], because it occurs on the opposite side of the motor domain [11]. It was concluded that ELC interactions with the motor domain significantly change in the course of conformational transition from the S1 nucleotide-free state to the complexes S1-ADP-AlF_4^- or S1-ADP-BeF_x. In other words, the motor domain and ELC are involved in the interactions that differ in the position of the lever arm relatively to the motor domain (Fig. 3). These structural data indicate that the lever arm is not a simple mechanical device. On the contrary, interactions between ELC in the regulatory domain and motor domain of the myosin head during the ATPase cycle are likely to play an important role in the functioning of the myosin head as a molecular motor.

The data on the atomic structure of S1 and modeling of certain stages of the ATPase reaction have indicated that rotation of the regulatory domain results not only in its rapprochement to the motor domain, but also in a sufficiently strong interaction between these domains, more accurately, between the motor domain and ELC associated with the regulatory domain [11]. Although predicted in

[11], such interaction has not been experimentally proven yet. In particular, the authors could not solve the X-ray structure of ELC-bound nucleotide-free S1 due to methodical difficulties, so they had to compare the structures of the smooth muscle S1 in the triple S1-ADP-AlF_4^- and S1-ADP-BeF_x complexes [11] with the earlier resolved structure of the nucleotide-free S1 from skeletal muscles [3].

Interesting results were obtained by studying polarized fluorescence of S1 containing ELC fluorescently labeled at Cys180 in its *C*-terminal part [53]. It was shown that the ELC *C*-terminal part was noticeably more immobilized and ordered in the presence of ATP than in the absence of nucleotides. No similar effects were observed when S1 was modified at Cys707 in the motor domain or when ADP or PP_i were used instead of ATP. The authors believe that these results show that ATP binding to the active site in S1 affects the ELC mobility, probably, because of the ELC interaction with the S1 motor domain resulting in partial immobilization of the fluorescent label [53].

The fact that the *C*-terminal part of ELC in S1 is immobilized stronger in the presence of ATP is unexpected, because it contradicts the earlier hypothesis on the interaction between the S1 motor domain heavy chain and the *C*-terminal part of ELC in the regulatory domain of the nucleotide-free S1 [52]. Note that this hypothesis was based on the crystal structure of S1. However, recent data of high resolution microscopy have shown that even in the absence of nucleotides, the S1 regulatory domain has a sufficiently high mobility and can rotate relatively to the motor domain [54]. It may be assumed that in the presence of ATP or in the S1-ADP-BeF_x complex, the mobility of the S1 regulatory domain is significantly decreased because of the interactions between the S1 regulatory domain (more accurately, ELC associated with it) and motor domain.

The hypothesis that the myosin head regulatory domain interacts with the motor domain during the ATPase cycle required experimental confirmation. In our laboratory, we have studied the S1 domain structure and its changes in the stable S1-ADP-V_i, S1-ADP-BeF_x and S1-ADP-AlF_4^- triple complexes using differential scanning calorimetry (DSC) combined with other approaches. DSC allows to register structural rearrangements in the myosin head during formation of the S1 complexes with ADP and P_i analogs (manifested as sharp increases in the S1 thermostability) [55, 56] and changes in the S1 domain structure [56-58].

Analysis of the myosin head domain structure by DSC is especially interesting because interdomain interactions induce pronounced intramolecular translocations in the myosin head and play the key role in the transformation of the ATP hydrolysis energy into mechanical work. DSC is one of the best approaches for detecting structural domains in multidomain proteins as individual thermal transitions on the heat absorption curves. Such

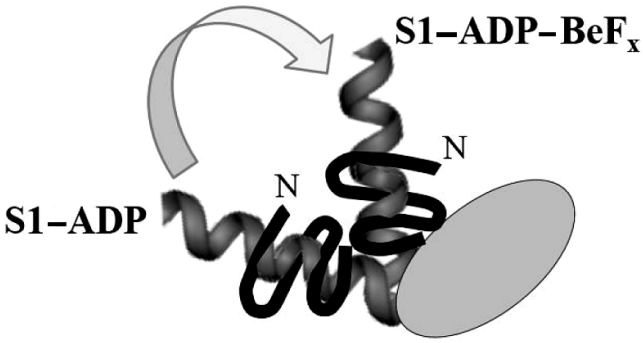

Fig. 3. Schematic representation of the rotation of the S1 regulatory domain containing ELC (black line) relatively to the motor domain (grey oval) in the course of the S1-ADP-BeF_x triple complex formation. See the text for details.

domains (named calorimetric domains) can be defined as regions in the protein molecule that fold cooperatively and independently from each other [59]. Earlier studies have found three calorimetric domains in the nucleotide-free S1 molecule [55, 57, 60]. Later, the mechanism of S1 thermal denaturation was revised. In particular, it was shown that S1 has two (not three) calorimetric domains [61]; denaturation of the more thermostable domain occurs in two stages (thermal transitions 2 and 3 in Fig. 4a). To localize calorimetric domain to particular regions in the S1 molecule, the DSC data were analyzed together with temperature dependences of the S1 intrinsic tryptophan fluorescence. It is known that all tryptophan residues of S1 are present only in the S1 motor domain; therefore, changes in the S1 fluorescence will be observed within the temperature range corresponding to the motor domain denaturation. Irreversible thermal denaturation of S1 was accompanied by the tryptophan fluorescence spectrum shift to shorter wavelengths and, therefore, changes in the parameter $A = I_{320}/I_{365}$ (the ratio of fluorescence intensities at the corresponding wavelengths). Initially, this parameter was introduced for the assessment of the native state of actin [62]. Comparison of the temperature dependences for the completeness of three calorimetric transitions of S1 with the temperature dependence of the normalized parameter *A* (completeness of transition by the parameter *A*) (Fig. 4b) showed that *A* changed in the regions corresponding to calorimetric transitions 2 and 3, but not in the region of calorimetric domain 1 denaturation (Fig. 4b). Based on these data, we suggested that the more thermostable calorimetric domain including thermal transitions 2 and 3 corresponded to melting of the S1 motor domain, whereas the less thermostable calorimetric domain 1 corresponded to melting of the tryptophan-free regulatory domain [61].

Similar experiments were performed for S1 in the S1-ADP-BeF_x and S1-ADP-AlF_4^- triple complexes [63]. The fact that the S1 molecule in the S1-ADP-BeF_x com-

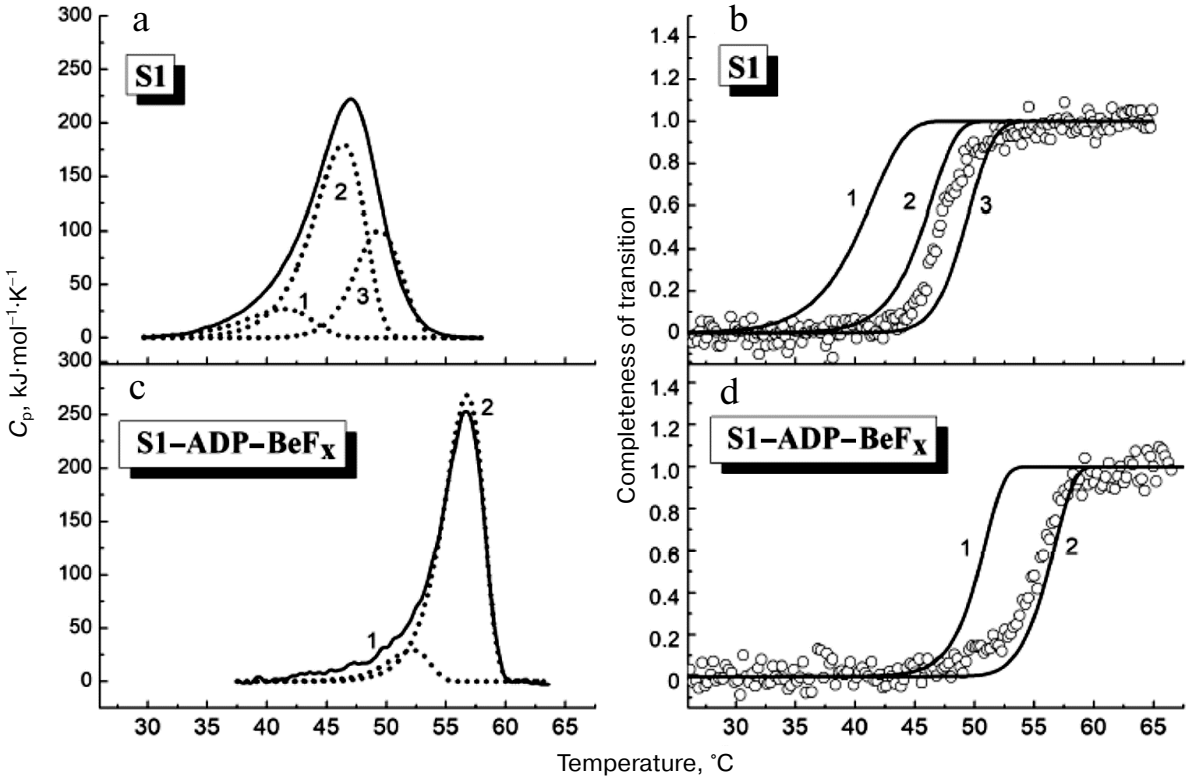

Fig. 4. Thermal denaturation of S1 in the absence of nucleotides (a, b) and in the triple complex S1-ADP-BeF_x (c, d). a, c) Temperature dependences of the excess heat capacity obtained by the DSC method for S1 in the absence of nucleotides (a) and in the S1-ADP-BeF_x complex (c), and their deconvolution into individual thermal transitions (calorimetric domains). b, d) Comparison of the temperature dependences of the normalized parameter *A* (circles) characterizing changes in the S1 tryptophan fluorescence in the absence of nucleotides (b) and in the S1-ADP-BeF_x complex (d) with the completeness of the thermal transitions corresponding to the calorimetric domains in the S1 molecule (solid lines; numerals denote the numbers of the calorimetric domains). Conditions: 30 mM Hepes, 2 mM $MgCl_2$, 100 mM NaCl, pH 7.3. The heating rate was 1°C/min. See the text for details.

plex is represented by two calorimetric domains that melt at high temperatures (~52 and 56-57°C) was established in the early DSC studies of the S1 domain structure [58]. The same two domains were observed in more recent studies of the S1-ADP-BeF_x (Fig. 4c) and S1-ADP-AlF_4^- complexes [63]. Comparison of the DSC data presented in Figs. 4a and 4c reveals no increase in the heat sorption at 35-45°C in the S1-ADP-BeF_x complex, i.e., in the temperature range for the S1 regulatory domain melting in the absence of nucleotides (Fig. 4, a and b). This means that in the S1-ADP-BeF_x complex, S1 regulatory domain is likely to denature within some other temperature range. Comparison of the DSC data with the temperature dependence of tryptophan fluorescence showed that fluorescence of S1 in the S1-ADP-BeF_x complex changes in the interval of denaturation of the more thermostable domain 2, but not in the interval of denaturation of the less thermostable domain 1 (Fig. 4d). It was suggested that calorimetric domain 1 (Fig. 4, c and d) in the S1-ADP-BeF_x complex reflects thermal denaturation of the S1 tryptophan-free regulatory domain. In the complexes S1-ADP-BeF_x and S1-ADP-AlF_4^-, this domain melts at a higher temperature (approximately by 10°C) than in the absence of nucleotides, which might be due to its interaction with the S1 motor domain [63].

This suggestion required experimental verification; in particular, it was necessary to identify thermal transitions in the DSC thermograms to elucidate which transition corresponds to the melting of the S1 regulatory domain in the absence of nucleotides and in the complexes with ADP and P_i analogs. It should be noted that in the above-described studies, such identification was made by indirect methods and was based on the assumption that all tryptophan residues were present only in the S1 motor domain and absent from the regulatory domain and regulatory domain-associated ELC. It would be much more interesting to obtain direct information on the thermal denaturation of the regulatory domain itself. Such information could be obtained, for instance, from the temperature dependences of fluorescence of a labeled ELC associated with the S1 regulatory domain and by comparing these dependences with the DSC data on the S1 domain structure in the absence of nucleotides and in complexes with ADP and P_i analogs.

Such experiments were performed at the next stage of our studies. Recombinant ELC isoforms (A1) were

obtained in which Cys180 was replaced with Ala and then SH-groups were introduced in different areas of the *C*-terminal part (E160C, S142C, T127C, or S99C) thereby "moving" the SH-group in ELC further from the *C*-terminus. The recombinant ELCs were modified with a fluorescent label (1,5-IAEDANS) and used to replace native ELCs in S1 by incubating S1 with an 8× molar excess of the modified ELC at 37°C in the presence of 10 mM MgATP (for details, see [63]). Then, we recorded temperature dependences of the fluorescence of the label attached to the introduced SH-groups in the *C*-terminal part of ELC associated with the S1 regulatory domain and compared them to the temperature dependence of tryptophan fluorescence characterizing thermal denaturation of the S1 motor domain and the DSC data on the changes in the domain structure of S1 in the S1-ADP-AlF_4^- and S1-ADP-BeF_x triple complexes. This allowed us to identify thermal transition corresponding to the S1 regulatory domain in the DSC thermograms. We confirmed that in the triple complexes, this domain denatured at a significantly higher temperature than in the absence of nucleotides [63].

All these data corroborated the hypothesis that the ATPase reaction is accompanied by tight interactions between the two major domains of the myosin head – the motor domain and the regulatory domain (or more accurately, between the motor domain and ELC associated with the regulatory domain), that dramatically change the character of the regulatory domain thermal denaturation by increasing its thermostability.

Very recently, we have obtained an additional confirmation of this hypothesis by using Forster resonance energy transfer (FRET) to determine the distance between two fluorescent labels (fluorescence energy donor and acceptor) attached to residues in the different areas of the protein molecule. The distances determined were from the ELC residues Cys99, Cys160, or Cys180 modified with 1,5-IAEDANS (donor) to the ATPase active site in the S1 motor domain with the bound fluorescent ADP analog TNP-ADP (acceptor) [64]. We found that formation of the S1-ADP-BeF_x complex was accompanied by a dramatic decrease in these distances, the effect being more pronounced in the case of fluorescently labeled Cys99 and Cys160, when the distance to TNP-ADP in the S1 active site decreased nearly twofold (from 5.7-6.5 nm and over for S1-ADP to 3.3-3.7 nm for S1-ADP-BeF_x) [64].

FUNCTIONS OF THE ELC *N*-TERMINAL EXTENSION

ELC isoforms containing the *N*-terminal extension (A1) are expressed in both skeletal and cardiac muscles; however, only in fast skeletal muscles these isoforms are synthesized together with the shorter A2 isoforms. S1 preparations obtained by hydrolysis of fast skeletal muscle myosin with chymotrypsin and lacking RLC can be fractionated by ion-exchange chromatography into S1(A1) and S1(A2) fractions containing ELC A1 or A2 isoforms, respectively [18, 19]. These fractions are often used to study the functional role of the *N*-terminal extension of A1.

Interactions of the *N*-terminal extension of A1 with actin. Undoubtedly, the major function of the *N*-terminal extension of A1 is its interaction with actin. Even in the earliest works, it was shown that actin stimulates the ATPase activity of S1(A1) to a lesser extent than the ATPase activity of S1(A2), but the affinity of S1(A1) for actin is significantly higher than the affinity of S1(A2) [18]. The high affinity of S1(A1) for actin was confirmed by affinity chromatography on immobilized actin: S1(A2) was eluted from the column with a Mg-ATP concentration gradient before S1(A1) [65]. It is important to note that all these differences in the properties of S1(A1) and S1(A2) were observed only at low ionic strength (6 mM KCl) but disappeared with the ionic strength increase to 46 mM. The increase in the ionic strength did not affect the actin-stimulated ATPase activity of S1(A2) but elevated the ATPase activity of S1(A1) to the values typical for S1(A2) [66]. Similar differences between S1(A1) and S1(A2) were observed in the affinity of their binding to F-actin. When co-precipitated with actin in the presence or absence of ATP at a low ionic strength, S1(A1) bound actin significantly stronger than S1(A2), but this difference completely disappeared with an ionic strength increase [67]. It is interesting to note that in all these cases, the changes in the ionic strength affected to properties of S1(A1) and shifted them toward the properties of S1(A2). Therefore, the existence of differences between S1(A1) and S1(A2) can be explained by the presence of the *N*-terminal extension in A1.

By now, a large body of evidence has been obtained that the *N*-terminal extension of A1 directly interacts with actin. The first experimental confirmation of this interaction was obtained by ^{1}H-NMR. The authors [68] showed that the S1(A1) interaction with actin was accompanied by pronounced structural changes in the *N*-terminal extension of A1. Another proof of such interaction was obtained when the S1(A1) and S1(A2) complexes with actin were treated with a bifunctional reagent with the cross-link length equal to zero (water-soluble carbodiimide). It was shown that at low ionic strength, A1 (unlike A2) cross-linked to the region of residues 360-363 in the *C*-terminal part of actin molecule [69]. An increase in the ionic strength (100 mM KCl) decreased the efficiency of cross-linking by 50% [70]. In 1985, researchers succeeded for the first time in isolating the *N*-terminal extension of A1 from rabbit skeletal muscles and bovine cardiac muscles by treating A1 with thrombin. Using affinity chromatography and ^{1}H-NMR spectroscopy, this fragment was shown to be capable of direct interaction

with actin [71]. Later, detailed analysis of three-dimensional images of actin filaments decorated with S1(A1) or S1(A2) obtained by cryoelectron microscopy clearly proved that the A1-binding site is located in the region of residues 360-363 in the *C*-terminal part of actin molecule [72].

The role of N-terminal Lys residues. It was shown by ^{1}H-NMR that only several first amino acid residues (Ala1-Pro2-Lys3-Lys4) of the *N*-terminal extension of A1 directly interact with actin [73]. Removal of the first 11 *N*-terminal residues (including two pairs of lysine residues) from the A1-like recombinant ELCa associated with S1 led to almost complete abolishment of its cross-linking with actin [33]. Treatment of myosin filaments with papain resulted in the S1(A1) preparation in which A1 was lacking the first 13 amino acids of its *N*-terminal extension [74]. The kinetic parameters of the actin-stimulated ATPase activity (K_m and V_{max}) of this preparation were very different from those of S1(A1) with intact A1, but closely resemble kinetic parameters of S1(A2). Moreover, the authors isolated the 13-a.a. *N*-terminal extension of A1 and showed that it was able to cross-link with actin. Addition of this fragment to S1(A1) changed the kinetic parameters of the actin-stimulated ATPase reaction by making them similar to the parameters characteristic for S1(A2) [74]. Based on these data, an interesting conclusion could be made on the competition for actin binding between the *N*-terminal extension of A1 in the content of S1(A1) and free *N*-terminal extension in the ATPase cycle. In its turn, this indicates that kinetic parameters of the actin-stimulated ATPase reaction of S1(A1) differ from the kinetic parameters of S1(A2) only due to the presence of the *N*-terminal extension in A1, in particular, due to presence of positively charged Lys residues at the *N*-terminus that interact with actin during the ATPase cycle. In [73], site-directed mutagenesis was used to obtain A1 mutants in which Lys residues at positions 3 or 4 were replaced with Ala or Lys at position 4 was replaced with Asp. It was shown that these replacements strongly decreased the extent of actin cross-linking to A1 in the content of S1(A1).

The importance of positively charged Lys residues at the *N*-terminus of the A1-like ELC from heart ventricles (ELCv) for the interaction with actin was shown in cardiac muscle fibers using synthetic peptides that corresponded to the ELCv residues 5-14, 5-10, and 5-8 [75]. Addition of these peptides to the muscle fibers increased their contraction/relaxation rate, the greatest effect being observed for the longest peptide corresponding to the fragment 5-14 including five Lys residues (control peptides with the random amino acid sequences did not influence the force and the rate of fiber contractions) [75]. The same peptide produced similar effects in rat cardiac myofibrils [76]: it competed with ELCv for actin binding and increased the ATPase activity of myofibrils nearly twofold.

The role of Ala-Pro repeats in the N-terminal extension of A1. Another segment of the *N*-terminal extension that plays an important role in A1 functioning is the region of Ala-Pro repeats (residues 14-27) (Fig. 1). The role of these repeats in the myosin interaction with actin was elucidated using the methods of protein engineering. Removal of this segment from the *N*-terminal extension with preserving the *N*-terminal Lys residues did not affect the ability of A1 to bind and cross-link to actin but completely abolished A1 cross-linking with actin if such shortened A1 was in the content of S1. In the latter case, the kinetic parameters of the actin-stimulated ATPase activity of S1 with the shortened A1 did not differ from those of S1(A2) [77]. Note that A1 with the duplicated region of Ala-Pro repeats could be efficiently cross-linked with actin in both free state and in the content of S1. The affinity for actin of the S1 preparation containing A1 with the duplicated region of Ala-Pro repeats was similar to that of S1(A1), whereas the maximal rate of the actin-stimulated ATPase reaction (V_{max}) was the same as for S1(A2) [77]. Therefore, duplication of the Ala-Pro repeats in A1 did not affect the ability of A1 in the content of S1(A1) to bind actin, but completely abolished the effect of such interaction on the rate of ATP hydrolysis in the active site of S1. This means that the Ala-Pro repeats can not only form an elongated structure that allows positively charged *N*-terminal Lys residues in A1 to "reach out" to the actin surface but also influences the interaction of the *N*-terminal extension with actin.

According to the current concepts based on the structural modeling, the semi-rigid antenna-like structure of the region of Ala-Pro repeats allows this region to act as an extended "bridge" connecting the *C*-terminal part of A1 associated with the myosin head regulatory domain and the binding site for the positively charged *N*-terminus of A1 in the actin filament [21, 22]. How the *N*-terminal extension of A1 spans the distance from the myosin head regulatory domain to the actin surface is not yet known. FRET measurements have shown that in the free-state A1, the distance between the first and last residues of the *N*-terminal extension is ~4.1 nm, and this distance does not change under denaturing conditions in the presence of 6 M guanidine hydrochloride. This suggests that in the free-state A1, the *N*-terminal extension is a statistical coil [78] that forms a semi-rigid elongated structure only upon S1(A1) interaction with actin. Computer-assisted docking of the S1 structure into the three-dimensional reconstruction of actin filaments decorated with S1 allowed to evaluate the distance from the first permitted region in the ELC structure in S1 (residue 5 in A2) to the nearest region on the actin surface (>8 nm) [78]. Moreover, this region was found not on the actin monomer bound to the S1 heavy chain but on the neighboring monomer in the actin filament.

The fact that the heavy chain of S1 and the *N*-terminal extension of A1 bind to different actin monomers dur-

ing S1(A1) interaction with actin filaments has been proven using different approaches, e.g., cross-linking of the *N*-terminal extension of A1 with actin at different molar ratios between S1(A1) and actin [79]. An increase in this ratio initially leads to the increase in the amount of A1 cross-linked to actin; however, as the ratio approaches 1 : 1, the reaction of cross-linking slows down until complete stop at full saturation of actin filaments with S1(A1) molecules. This can be explained by a higher affinity of the S1 heavy chain for actin than of the *N*-terminus of A1, so that at high saturation of the actin filaments with myosin heads, all actin monomers are occupied with the S1 heavy chains instead of the *N*-terminal extensions of A1. Cross-linking of the S1 heavy chain and the *N*-terminal extension of A1 with different actin monomers was confirmed in [33], in which the authors used DNase I with a high affinity for monomeric actin (G-actin) that prevented its polymerization into actin filaments (F-actin). Addition of free A1 to the G-actin complex with DNase I resulted in the formation of cross-links between actin and the *N*-terminal extension of A1. In the presence of S1(A1), only the S1 heavy chain interacted with actin monomers.

It was also shown that S1(A1) could initiate actin polymerization, i.e., formation of F-actin filaments from monomeric G-actin, at significantly lower concentrations and more rapidly than S1(A2) [80]. In [78], a mutant form of A1 lacking the Ala-Pro repeats was obtained. It was shown that S1(A1) containing this mutant form of A1 lost its ability to rapidly polymerize actin and its behavior became similar to that of S1(A2). Also, the ability S1(A1) with the mutant A1 to polymerize actin correlated with the kinetic parameters of the actin-stimulated ATPase activity that were identical to those of S1(A2) at low ionic strength (5 mM KCl). Therefore, the ability to interact with actin in the ATPase cycle and stimulation of actin polymerization are determined by the interactions of the *N*-terminus of A1 with actin. Based on these data, it may be assumed that the S1 heavy chain and the *N*-terminus of A1 bind to different actin monomers, which provides faster and more efficient F-actin polymerization.

Therefore, due to the semi-rigid antenna-like structure of the Ala-Pro repeats, the major function of this region in the interaction of S1(A1) with actin is to make it possible for the *N*-terminal positively charged Lys residues in A1 to reach the cluster of *C*-terminal acidic residues 360-363 in the actin monomer other than the monomer bound to the S1 motor domain. Concurrently, an additional actin-binding site is formed on the myosin head that significantly increases head affinity for actin.

What is the role of the ELC *N*-terminal extension in the molecular mechanism of muscle contraction? Facts and hypotheses. First of all, it is necessary to answer the question whether the *N*-terminal extension of A1 interacts with actin and functions at physiological values of ionic strength. This question has been under discussion for a long time mostly due to the fact that the majority of earlier studies comparing the properties of S1(A1) and S1(A2) showed that at low ionic strength, the affinity of S1(A1) for actin is significantly higher than that of S1(A2). However, this difference completely disappears at ionic strength values close to the physiological ones (~100-140 mM) [18, 65-67]. The electrostatic character of the interactions between positively charged Lys residues at the *N*-terminus of A1 and negatively charged actin residues easily explains this. On the other hand, the data have been accumulated that the *N*-terminal extension of A1 interacts with actin filaments even at high ionic strength values close to the physiological ones. Thus, cross-linking of A1 with actin was observed also at physiological values of ionic strength (~100 mM KCl), although the efficiency of cross-linking was 50% lower than that at low ionic strength [70]. Experiments in rabbit slow muscle fibers showed that substitution of A1 with A2 in myosin resulted in a pronounced increase in the fiber contraction rate that depended on the ratio between A1 and A2 even at a high ionic strength (120-180 mM) [81].

Recently, we obtained new evidence that the *N*-terminal extension of A1 interacts with F-actin at physiological values of ionic strength [82]. Using FRET, we determined the distance from Cys374 modified with 1,5-IAEDANS (donor) in actin to the Cys residues at different positions in the *N*-terminal extension of A1. These residues were introduced by the site-directed mutagenesis and then modified with 5-IAF (acceptor). It was shown that at the physiological ionic strength (120-150 mM NaCl) and the S1(A1) to actin molar ratio of 1 : 3 (i.e., under conditions allowing interaction of the *N*-terminal extension with actin), these distances were 5.2 nm to Cys40, 3.7 nm to Cys15 located between the Ala-Pro repeats, and <2.5 nm to Cys6 located near the Lys residues in the *N*-terminus of A1. Such a short distance (<2.5 nm) from the *N*-terminus of A1 to actin confirmed the possibility of direct interaction of the *N*-terminal extension with actin under the conditions mentioned. All these distances significantly increased with the increase in the ionic strength to ⩾300 mM or at the S1(A1) to actin molar ratio of 1 : 1, i.e., under conditions preventing interaction of the *N*-terminal extension with actin [82]. Our data also proved that the interaction of the *N*-terminal extension with actin could occur at physiological values of ionic strength.

Direct confirmation of the important role of the *N*-terminal extension of the elongated ELC isoform in muscle contraction was obtained using transgenic mice that expressed a mutant form of human ELCv lacking the 43-a.a. *N*-terminal extension (i.e., similar to A2 from fast skeletal muscles) in the myocardium [83]. The replacement of ELC in the myocardium was not complete, so that the ratio between endogenous ELCv isoforms containing the *N*-terminal extension and mutant ELCv iso-

form was approximately the same as the ratio between A1 and A2 in fast skeletal muscles. The contractile force of muscle fibers isolated from the cardiac muscles of transgenic mice was essentially lower than that of the control fibers. It should be noted that the mutant mice had the cardiac muscle hypertrophy that, however, did not lead to serious functional disorders of the heart. The authors believe that the observed effects were due to the fact that myosin heads with the A1-like cardiac ELC isoform generate greater contractile force during one ATPase cycle than the heads with the A2-like isoform. Note that all these experiments were performed at high ionic strength values (150 mM) that were close to the physiological ones [83].

Other pieces of evidence proving interactions of the *N*-terminal extension of the A1-like ELC with actin in muscles have been obtained in transgenic animals. Thus, the authors of [84] studied whether expression of the *N*-terminal peptide of cardiac ELC could influence the contractility of cardiac muscle. For this purpose, they generated transgenic rats whose genome contained minigenes encoding peptides composed of 15 *N*-terminal amino acids of human ELCa or ELCv. Expression of these *N*-terminal peptides in cardiomyocytes noticeably affected the contractility of the isolated perfused hearts. The rate of the cardiac muscle contraction/relaxation in the transgenic rats was increased in comparison with the control line not expressing the *N*-terminal peptide [84].

Thus, A1- and A2-like ELCs have a different impact on the myosin interaction with actin. Due to its *N*-terminal extension, A1 provides an additional contact of the myosin head with actin during the ATPase cycle that allows it to generate a greater force per ATP used. By contrast, A2-containing myosin can develop a higher rate of contractions but with a lower contractile force. The fibers of cardiac and slow skeletal muscles contain only A1-like elongated ELC isoforms; smooth muscles express only short A2-like ELCs; the fibers of fast skeletal muscles produce both A1 and A2 isoforms. This distribution of ELC isoforms in muscle fibers is apparently not accidental. Cardiac myosin contains only A1-like ELC isoforms, which allows it to generate greater contractile force to provide the blood flow in the vessels. Smooth muscles that line the vessels and the intestine do not need such a high force of contraction; therefore, myosin heads in smooth muscles include the shortened isoform of ELC. Fast skeletal muscles have to contract at different rates and with different force; hence, they contain both A1 and A2 isoforms that can modulate these parameters.

An interesting hypothesis was proposed recently [85]. The authors attempted to give a new explanation to the role of the *N*-terminal extension of A1 in the mechanism of force generation by myosin heads during their interaction with actin in the ATPase cycle. They used time-resolved FRET (TR-FRET) to rapidly measure the distance between the myosin head and actin in the acto-myosin ATPase cycle, during the head transition from the weak actin binding state to the strong binding state and *vice versa*. The binding of S1(A1) and S1(A2) to actin was compared by measuring the distances from the fluorescent label attached to the *C*-terminal part of ELC common for A1 and A2 (Cys180 in A1 and Cys136 in A2) to the label attached to Cys374 in actin. It was shown that at weak S1 binding to actin, the distance between the labels was the same (9.6-9.7 nm) for both S1(A1) and S1(A2). Transition to strong actin binding significantly (by 1.7 nm) decreased the distance in S1(A1) but not in S1(A2). The authors' interpretation of these data is very interesting: at strong S1 binding to actin filament, interactions between the *N*-terminal extension of A1 with the neighboring actin monomer in the filament pull the regulatory domain of S1 closer to actin, thereby increasing the angle of its rotation relatively to the motor domain and, correspondingly, the force developed by the myosin head during such rotation [85].

Interaction of the *N*-terminal extension of A1 with the myosin head motor domain. Let's consider one of the most discussed questions concerning the functions of the ELC *N*-terminal extension, namely, whether this segment can interact with the motor domain of the myosin head. Interaction of the *N*-terminal extension of A1 with actin has been extensively studied; its existence does not cause any doubt, although some specific features of this interaction are still discussed. At the same time, the data on the interaction of this segment with the myosin head motor domain are scarce and often contradictory.

One of the first indications that the *N*-terminal extension of A1 interacts with the myosin head motor domain was that monoclonal antibodies against the first four *N*-terminal residues of A1 bind to the myosin head at a distance of ~11 nm from the site of its joining to the rod part of myosin, i.e., in the head motor domain, as demonstrated by electron microscopy [86]. Another indirect indication to such interaction is that chemical cross-linking of S1(A1) in the absence of actin strongly inhibits its actin-stimulated ATPase activity, whereas actin stimulation of the cross-linked S1(A2) remains unaffected [87]. More direct data on the chemical cross-linking were obtained by Pliszka et al. [88] who used a bifunctional reagent with the cross-link length equal to zero. The authors showed that the first 30 a.a. from the *N*-terminus of A1 could be cross-linked to the *C*-terminal fragment (*MM*, 20 kDa) of the S1 heavy chain and suggested that A1 was cross-linked with the so-called converter, a region of the S1 motor domain adjacent to the regulatory domain and containing numerous acidic residues capable of binding to the positively charged *N*-terminus of A1. The involvement of the *N*-terminus of A1 in this interaction was confirmed by the fact that removal of a ~2-kDa fragment from the *N*-terminus by S1(A1) treatment with thermolysine abolished the cross-linking. The efficiency of cross-linking was low, which suggested the weakness of

the interactions. It should be noted also that the cross-linking of the *N*-terminal extension of A1 with the S1 heavy chain was insensitive to ATP addition [88].

Lowey et al. [78] used cryoelectron microscopy to determine the position of the gold-labeled *N*-terminus of A1 in the content of S1 on actin filaments decorated with S1. Unexpectedly, the *N*-terminal extension of A1 was found in the vicinity of the SH3-domain located near the *N*-terminus of the myosin heavy chain in the S1 motor domain (residues 35-80) and not on actin. Taking into account that SH3-domains bind proline-rich ligands, the authors suggested that the proline-enriched region of the *N*-terminal extension of A1 could bind to the SH3-domain of S1 [78]. However, it should be noted that these experiments were performed on actin filaments fully decorated with S1, i.e., under conditions very far from the physiological ones, when the *N*-terminal extension of A1 cannot bind to actin at all [79].

The actual position of the *N*-terminal extension of A1 in the myosin head is unknown, because none of the known atomic structures of S1 has this segment due to one or another reason. In the S1 from chicken skeletal muscles, this region was degraded by papain [89] and therefore it was absent from the crystal structure [3]; A2-like ELCs in the S1 preparations from smooth muscles [11] and scallop muscles [6] lacked the *N*-terminal extension. To solve this problem, scientists have used computer-aided modeling. Thus, computer-aided docking of the S1 structure into the three-dimensional reconstruction of actin filaments decorated with S1 made it possible to evaluate the distance from the first permitted *N*-terminal residue in the ELC (residue 5 in the A2 sequence) to the nearest region on actin surface (8.9 nm) and to the SH3-domain of the S1 heavy chain (6.6 nm), as well as the distance from the SH3-domain to the actin surface (3.6 nm) [78]. Based on these data, the authors proposed that the proline-rich region of Ala-Pro repeats in the *N*-terminal extension of A1 is permanently bound to the SH3-domain of S1 at all stages of the ATPase cycle and that this segment of A1 uses the SH3-domain as a "starting area" for further interaction of the positively charged Lys residues at the *N*-terminus of A1 with actin [78].

Considering the aforementioned data, it is possible that the proline-enriched sequence of the *N*-terminal extension of A1 (residues 15-28) remains bound to the S1 motor domain independently of the presence of ATP. However, it appears untrue for the strongly charged *N*-terminus of A1, whose electrostatic interactions with actin and, probably, with the S1 motor domain depends on ATP. We believe that at different stages of the ATPase cycle, the *N*-terminus of A1 can bind either to actin (when S1 is strongly bound to actin) or to the S1 motor domain (when S1 binds ATP and dissociates from actin). This suggests that these two types of the *N*-terminus interactions (with actin and S1 motor domain) have to be ATP-dependent. The idea on the ATP-induced interaction of the *N*-terminus of A1 with the S1 motor domain was confirmed by the data on the polarized fluorescence of S1 containing A1 fluorescently labeled at the *N*-terminus that demonstrated a pronounced increase in the fluorescence anisotropy caused by ATP addition. The authors believe that the interaction of the *N*-terminus of A1 with the S1 heavy chain leads to a partial immobilization of the fluorescent label [53]. It may be assumed that under certain conditions, when the myosin head dissociates from actin in the ATPase cycle, the *N*-terminal extension of A1 can be involved not only in the intermolecular interactions with actin but also in the intramolecular interactions with the myosin head motor domain.

The hypothesis on the interaction of the *N*-terminal extension of A1 with the myosin head motor domain was confirmed by the ability of this extension to induce interactions between S1(A1) molecules resulting in their unusual aggregation described for the first time in [90, 91]. Later, the aggregational properties of S1(A1) and S1(A2) have been analyzed and compared more carefully [92, 93]; the two S1 isoforms were shown to be significantly different in the temperature dependences of their aggregation measured by the increase in the preparation apparent optical density. Thus, S1(A1) aggregated at a significantly lower temperature than S1(A2) [92, 93]. Aggregation of S1(A1) (but not of S1(A2)) strongly depended on the protein concentration – an increase in the protein concentration shifted aggregation to lower temperatures. This aggregation of S1(A1) did not depend on protein thermal denaturation and could occur even at low temperatures, although rather slowly [92].

The unusual aggregational properties of S1(A1) were studied in more detail by the method of dynamic light scattering (DLS) [93]. These experiments were performed at low ionic strength and relatively low temperature, that was below the temperature of S1 denaturation accompanied by spontaneous amorphous aggregation of the protein. Thermal denaturation of S1 isoforms was recorded in a series of experiments using differential scanning calorimetry (DSC). Heating of S1(A1) to 40°C did not cause S1 thermal denaturation but resulted in a pronounced increase (from ~18 to ~600-700 nm) of the hydrodynamical radius of particles, whereas in the case of S1(A2), the hydrodynamical radius remained unchanged (~18 nm). Similar differences between S1(A1) and S1(A2) were also observed in the presence of ADP. Note that S1(A1) aggregation was extremely sensitive to ionic strength and disappeared in the presence of 25 mM KCl. Moreover, S1(A1) aggregation induced by A1 was fully reversible – addition of 100 mM KCl to S1(A1) aggregates formed by the preparation heating to 40°C with subsequent cooling led to their complete dissociation accompanied by a decrease in the particle hydrodynamic radius from ~600 to 16-20 nm [93]. It was concluded that unusual aggregational properties of S1(A1) result from the intermolecular interactions of the *N*-terminal exten-

sion of A1 with other S1 molecules, supposedly, with their motor domains. These interactions do not depend on the S1 thermal denaturation and occur even at low temperatures; however, they become much more intensive with the temperature increase due to the Brownian motion that increases the probability of collisions between S1 molecules.

Similar experiments were performed with S1(A1) and S1(A2) in the S1-ADP-BeF_x and S1-ADP-AlF_4^- complexes using the DLS method. It was found that the S1(A1) and S1(A2) complexes did not differ in their temperature dependences of the hydrodynamic radius [93], which evidenced that formation of the triple S1-ADP-BeF_x or S1-ADP-AlF_4^- complexes completely prevented intermolecular interactions of the *N*-terminal extension of A1 manifested as unusual aggregational properties of S1(A1) at low ionic strength.

Based on these data, it was suggested that the *N*-terminal extension of A1 can interact with the motor domain of the same S1 molecule in the ATPase cycle, which explains why the aggregational properties of S1(A1) and S1(A2) in the S1-ADP-BeF_x and S1-ADP-AlF_4^- complexes do not differ. In the absence of nucleotides (or in the presence of ADP), the *N*-terminal extension of A1 interacts with actin thereby generating an additional actin-binding site in the myosin head. In the absence of actin, this extension is uncapable of intramolecular interaction but binds to the motor domain of another S1 molecule. These intermolecular interactions of the *N*-terminal extension of A1 can explain unusual aggregation properties of S1(A1) [93].

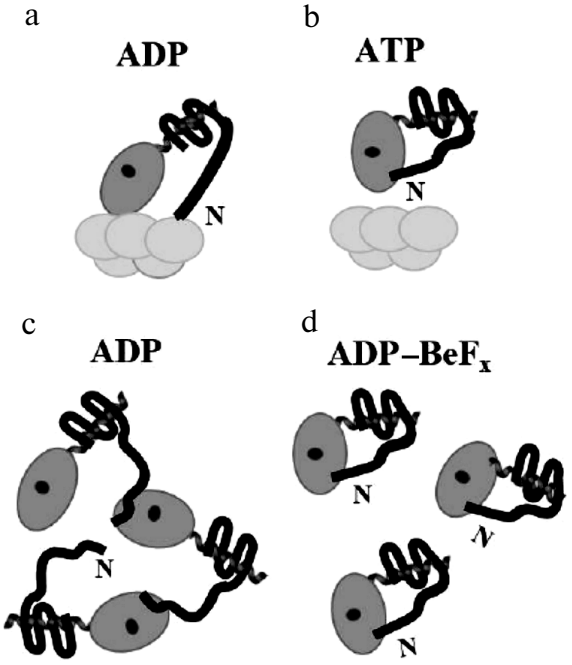

Fig. 5. Hypothetical intramolecular and intermolecular interactions of the *N*-terminal extension of A1 during the myosin head ATPase cycle in the presence and absence of actin. a, b) Interactions in the presence of actin: at the strong binding of the head to actin in the presence of ADP (a) and upon dissociation of the myosin head from the actin filament in the presence of ATP (b). c, d) Interactions in the absence of actin: intermolecular interactions of S1(A1) molecules in the presence of ADP (c) and intramolecular interactions in the S1(A1)-ADP-BeF_x complex (d). The A1 light chain and its *N*-terminal extension are shown with thick black line. See the text for details.

It should be noted that the above-presented data provide only indirect evidence in favor of interactions between the *N*-terminal extension of A1 and the S1 motor domain in the ATPase cycle. To obtain direct confirmations of such interactions, we used the FRET method. At first, we generated a number of recombinant A1 forms with an SH-group located in different regions of the *N*-terminal extension of A1 (V6C, A15C, S40C, and A57C) by site-directed mutagenesis. We also created an A1 mutant of with the Cys residue located virtually at the very *N*-terminus by introducing an additional CGI sequence immediately after the first two *N*-terminal residues (Met-Ala). In the mutant proteins, Cys180 in the *C*-terminal part of A1 was replaced with Ala. The recombinant A1 proteins were modified with 1,5-IAEDANS (donor) and used for replacement of native ELCs in the S1 molecules [64]. A fluorescent analog of ADP (TNP-ADP) was used as an acceptor located in the active site of S1 ATPase. Formation of the S1-ADP-BeF_x complex (stable analog of the S1 ATPase reaction intermediate S1*-ATP) resulted in a significant decrease in the distance from the Cys residues in the *N*-terminal extension of A1 and TNP-ADP in the S1 active site. The effect was especially pronounced when the labeled Cys residue was close to the *N*-terminus of the protein: for these residues the distance to TNP-ADP decreased from 5.7 to 3.5-3.7 nm upon formation of the S1-ADP-BeF_x complex [64]. The results of these experiments favor the earlier hypothesis that the *N*-terminal extension of A1 interacts with the myosin head motor domain during the ATPase reaction [64]. We believe that such interaction occurs in the myosin head only during the ATPase cycle (in our case, in the complexes S1-ADP-BeF_x or S1-ADP-AlF_4^- mimicking the S1 ATPase cycle intermediates S1*-ATP and S1**-ADP-P_i).

In conclusion, let's consider intramolecular and intermolecular interactions that involve the *N*-terminal extension of A1 during the myosin head ATPase cycle in the presence and absence of actin (Fig. 5). When myosin strongly binds actin and the myosin head is free of nucleotide or contains bound ADP, the *N*-terminal extension of A1 interacts with the actin filament (with the actin monomer adjacent to the monomer bound to the head motor domain). This generates an additional actin-binding site on the myosin head and makes stronger its binding to the actin filament (Fig. 5a). When the myosin head binds ATP, it undergoes conformational rearrangements and dissociates from the actin filament. Obviously,

the *N*-terminal extension of A1 also has to retire from actin; its contacts with actin are prevented during ATP binding and hydrolysis. We suggest that this is realized through the intramolecular interaction of the *N*-terminal extension of A1 with the myosin head, most likely with its motor domain (Fig. 5b). In the presence of actin, the *N*-terminal extension of A1 interacts with either actin (in the case of strong myosin binding to actin in the absence of nucleotides or in the presence of ADP) or with the myosin head motor domain (when it binds ATP and dissociates from actin).

Let's consider what happens with the *N*-terminal extension of A1 in an S1 solution in the absence of actin (Fig. 5, c and d). In the absence of nucleotides or in the presence of ADP, this segment, or at least its positively charged *N*-terminus, are located too far from the region in the motor domain of the same S1 molecule with which they can interact during the ATPase cycle. However, the *N*-terminal extension of A1 can bind to the similar region in another S1 molecule and cause S1(A1) aggregation [93] (Fig. 5c). This aggregation is completely prevented in the S1-ADP-BeF_x complex (Fig. 5d) mimicking the intermediate state of the ATPase reaction of S1 (S1*-ATP), i.e., the state in which the *N*-terminal extension of A1 supposedly interacts with the myosin head motor domain in the actomyosin ATPase cycle (Fig. 5b). This intramolecular interaction that might play an important role in the myosin head functioning as a molecular motor appears to be caused by global conformational rearrangements in the myosin head that occur during the ATP binding and hydrolysis and bring together the *N*-terminal extension of A1 and the myosin head motor domain as a result of the regulatory domain rotation relatively to the motor domain.

Acknowledgments

The work was performed within the framework of the State Task Section of the Federal Research Center "Fundamentals of Biotechnology", Russian Academy of Sciences (project 01201351368) and was supported by the Russian Foundation for Basic Research (project 18-34-00804-mol_a).

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