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40-kDa Actin-Binding Protein of Thin Filaments of the Mussel *Crenomytilus grayanus* Inhibits the Strong Bond Formation between Actin and Myosin Head during the ATPase Cycle

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Abstract—Mobility and spatial orientation of a novel 40-kDa actin-binding protein from the smooth muscle of the mussel *Crenomytilus grayanus* was studied by polarized fluorometry. The influence of this protein on orientation and mobility of the myosin heads was investigated during modeling the different stages of the ATPase cycle. The 40-kDa actin-binding protein affected the strong actin–myosin binding. We suggest that the 40-kDa actin-binding protein is involved in regulation of the actin–myosin interaction in the smooth muscle of the mussel.

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Key words: actin-binding protein of the mussel *Crenomytilus grayanus*, ghost fibers, ATP analog, actomyosin intermediate states, conformational change of myosin subfragment-1, fluorescence polarization

Calponins are multifunctional proteins involved in modulation of actin functions in smooth muscles and non-muscle cells [1]. In vertebrates three genes encode three calponin isoforms: alkaline h1-, neutral h2-, and acidic h3-calponin [2]. The alkaline h1-calponin binds to skeletal and smooth muscle actin [3]. Since the h1-calponin binding to actin results in inhibition of actomyosin Mg^{2+}-ATPase *in vitro* [4-9], it was supposed that this calponin isoform plays an important role in regulation of the actin–myosin interaction.

It was recently found that calponin enhances attachment of actin filaments to thiophosphorylated smooth muscle myosin [10]. Also, considering data on calponin binding to smooth muscle actin, Szymanski and Goyal [11] supposed that calponin could interact with the smooth muscle myosin regulatory light chain. They hypothesized that calponin, when inhibiting the actomyosin ATPase, can regulate the ATPase cycle by interacting not only with actin, but also with the myosin light chains, thus contributing to regulation of smooth muscle contractility.

Calponin-like proteins of invertebrates have recently attracted the attention of researchers. It was found, in particular, that the calponin-related protein UNC-87 from *Caenorhabditis elegans* antagonizes ADF (actin-depolymerizing factor)/cofilin-mediated actin filament dynamics [12]. Myophilin, a calponin-like protein from *Echinococcus granulosus*, undergoes phosphorylation by protein kinase C and possibly participates in regulation of smooth muscle contraction [13]. Another 38.3-kDa calponin-like protein from *Schistosoma japonicum*, which is localized in smooth muscle fibrils and associated with myofilaments and sarcoplasmic reticulum, is probably involved in cercaria tail muscle contraction [14].

Until quite recently only one calponin-like protein was known in bivalves. This is a 45-kDa protein isolated from the anterior byssus retractor muscle (ABRM) of the mussel *Mytilus galloprovincialis* [15]. Like calponin of vertebrates, this protein inhibits actomyosin Mg^{2+}-ATPase activity. The authors suggested that the 45-kDa calponin-like protein is implicated in regulation of smooth muscle contraction in bivalves.

We recently discovered a 40-kDa actin-binding protein in smooth catch muscle thin filaments of the mussel

Abbreviations: acrylodan, 6-acryloyl-2-dimethylaminonaphthalene; A·M, actomyosin; DTT, dithiothreitol; 1,5-IAEDANS, *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; TEMED, tetramethylethylenediamine.

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Crenomytilus grayanus. This protein also inhibits the actin-activated Mg^{2+}-ATPase of skeletal muscle myosin [16]. Biochemical and biophysical properties of this protein are similar to the properties of vertebrate smooth muscle calponin. The protein was named as 40-kDa calponin-like protein [16].

In the present work we studied the effect of the 40-kDa calponin-like protein on the structural state of actomyosin in muscle ghost fibers, when modeling some stages of the ATPase cycle with MgADP and MgATP nucleotides. The data suggest that the 40-kDa protein from the mussel – by changing its mobility and position on thin filaments – inhibits formation of the so-called strong actin–myosin bond in the skeletal muscle that is necessary for force generation.

MATERIALS AND METHODS

Preparation of proteins. Myosin subfragment-1 (S1) was prepared by digestion of rabbit skeletal muscle myosin with α-chymotrypsin at 25°C for 20 min according to the method of Okamoto and Sekine [17]. Thus prepared myosin subfragment-1 does not contain myosin regulatory light chains (Fig. 1, lane *1*). Cys707 of S1 was labeled with the fluorescent probe 1,5-IAEDANS [18] with protein/probe molar ratio 1 : 1 at 6°C for 24 h (Fig. 1, lane *3*). The reaction was terminated by addition of excess dithiothreitol (DTT). Unbound dye and excess DTT were removed by dialysis against 25-mM Tris-HC1 buffer, pH 7.0, containing 10 mM KCl, 1 mM $MgCl_2$, 1 mM NaN_3, and 0.1 mM DTT at 4°C. The dialysis buffer was replaced twice at 3 h intervals at protein/buffer ratio of 1 : 500 followed by a final 12-h dialysis step at protein/buffer ratio of 1 : 1000. The modification degree of S1 was calculated using the extinction coefficient $\varepsilon_{336} = 6100\ M^{-1}\cdot cm^{-1}$ [19]. The 40-kDa thin filament protein was isolated from the smooth catch muscle of the mussel *Crenomytilus grayanus*, as described earlier [16]. This protein was modified with 1.5-fold excess of the fluorescent dye acrylodan in 50-mM Tris-HCl buffer, pH 7.0, containing 100 mM NaCl at 6°C for 24 h; the protein concentration was 1 mg/ml [20]. The reaction was terminated by addition of excess DTT. The unbound dye and excess DTT were removed by exhaustive dialysis with the same buffer lacking acrylodan. The probe/protein molar ratio was estimated using the extinction coefficient $\varepsilon_{387} = 16{,}400\ M^{-1}\cdot cm^{-1}$ [21]. The acrylodan/calponin-like protein molar ratio was 0.9 : 1.0.

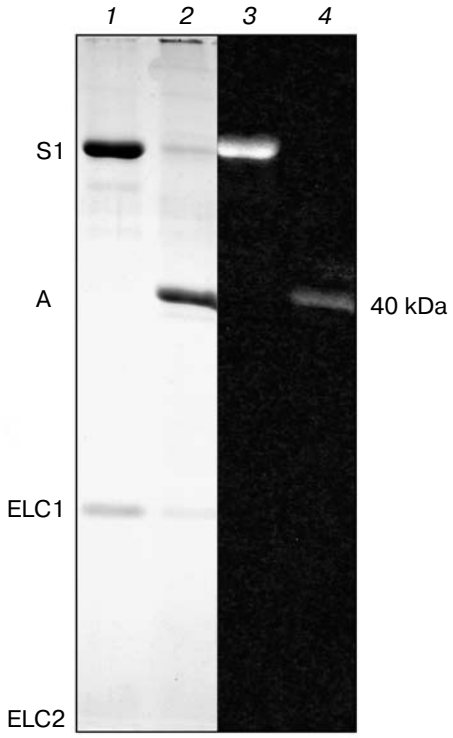

Fig. 1. SDS-PAGE of ghost fibers and S1 labeled with 1,5-AEDANS. S1, myosin subfragment-1; A, actin monomer; 40-kDa, the 40-kDa actin-binding protein; ELC1, essential light chain of myosin-1; ELC2, essential light chain of myosin-2. Lanes: *1*) S1-1,5-AEDANS; *2*) muscle ghost fibers decorated with S1 and containing the actin-binding protein labeled with acrylodan; *3*) S1-1,5-AEDANS under UV illumination; *4*) muscle ghost fibers decorated with S1 and containing the actin-binding protein labeled with acrylodan under UV illumination.

SDS-PAGE. Protein composition of muscle ghost fibers and specimens of S1, S1-AEDANS and 40-kDa protein labeled with acrylodan (40-kDa protein–acrylodan) were assayed by SDS-PAGE [22]. The stacking and resolving gels contained 4% and 10% polyacrylamide, respectively. Polymerization of gels was initiated by sequential addition of TEMED and ammonium persulfate solutions. The proteins were separated in 90 × 60 × 1.5 mm gel slabs in Tris-glycine buffer, pH 8.3, at current 30 mA per slab for 2.5-3 h. The gel was stained with bromophenol blue. The S1 to actin and calponin-like protein to actin molar ratios in ghost fibers were 1 : 2 and 1 : 1, respectively.

Isolation of ghost fibers. Ghost fibers were isolated by selective extraction of thick filament proteins, tropomyosin, and troponin from rabbit *m. psoas* for 1.5 h in 67 mM phosphate buffer, pH 7.0, containing 0.8 M KCl, 1 mM $MgC1_2$, and 5 mM ATP [23].

Decoration of ghost fibers. Myosin subfragment-1 (S1), S1-AEDANS, and the 40-kDa actin-binding protein (intact and acrylodan-labeled) were attached to thin filaments by incubation of the muscle thin filament in 10-mM Tris-HCl buffer, pH 6.8, containing 10 mM KCl, 1 mM $MgCl_2$, 0.1 mM DTT, and 1-2.5 mg/ml protein [24]. The unbound protein was removed and a single muscle fiber was washed for 5-8 min with the same solution containing no protein in a flow cell for removal of unbound protein.

Polarized fluorimetry. Polarized fluorescence of 1,5-IAEDANS and acrylodan was excited at 407 ± 5 and 479 ±

5 nm, respectively, and recorded at 550-650 nm. The measurements were carried out in 6.7-mM phosphate buffer, pH 7.0, containing 10 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, and 4 mM EGTA, either in the absence of nucleotides or in presence of 3 mM MgADP or 5 mM MgATP. Four intensities of polarized fluorescence, $_{\parallel}I_{\parallel}$, $_{\parallel}I_{\perp}$, $_{\perp}I_{\perp}$, and $_{\perp}I_{\parallel}$, where the left subscripts denote the parallel ($_{\parallel}$) and perpendicular ($_{\perp}$) direction of the exciting light polarization and the right subscripts the direction of the fluorescence polarization, were recorded using a polarization fluorimeter [25]. Fluorescence polarization degree P of fibers oriented parallel ($P_{\parallel}$) and perpendicular ($P_{\perp}$) to the plane of excitation light polarization was calculated from the equations: $P_{\parallel} = (_{\parallel}I_{\parallel} - _{\parallel}I_{\perp})/(_{\parallel}I_{\parallel} + _{\parallel}I_{\perp})$; $P_{\perp} = (_{\perp}I_{\perp} - _{\perp}I_{\parallel})/(_{\perp}I_{\perp} + _{\perp}I_{\parallel})$.

The experimental data were analyzed using a model-dependent method [26]. Suppose two fluorophore populations are in the muscle fiber: one spirals along thin actin filaments, with absorption and emission oscillator orientation angles Φ_A and Φ_E, respectively, and another (*N*) is chaotically oriented. Since in all experiments Φ_A changed similarly to Φ_E, Φ_A values are not shown. In the absence of nucleotides, the A·M stage of ATP hydrolysis was modeled. MgADP and MgATP were used for modeling the strongly bound (A·M·ADP) and weakly bound (A·M·ADP·Pi) actomyosin states, respectively [23]. According to the model, the angle Φ_E is an index of 1,5-IAEDANS and acrylodan orientation in the fiber, whereas *N* value depends on their mobility [27]. Statistical significance of changes was estimated using Student's *t*-test.

RESULTS AND DISCUSSION

Polarized fluorescence of 1,5-AEDANS, which is specifically bound to Cys707 of myosin motor domain, has been successfully used for more than 30 years for studying myosin structural states during muscle contraction [18, 28, 29]. The specimen of S1 modified with 1,5-AEDANS is shown in Fig. 1 (lanes *1* and *3*).

Decoration of S1-AEDANS thin filaments results in appearance of polarized fluorescence of muscle ghost fibers. By recording fluorescence of the fiber parallel and perpendicular to the polarization plane of the exciting light, we measured the fluorescence polarization degrees $P_{\parallel}$ and $P_{\perp}$, respectively. If emission oscillator dipoles are mainly parallel to the muscle fiber axis, and the fiber runs parallel to the polarization plane of the exciting light, then $P_{\parallel}$ is greater than $P_{\perp}$ [18, 29].

Table 1 shows that $P_{\parallel}$ is greater than $P_{\perp}$ in the absence of both nucleotides and the 40-kDa actin-binding protein, hence light emission dipoles of the dye bound with myosin heads are mainly in line with the muscle fiber. Analysis of the polarized fluorescence of S1 labeled with 1,5-AEDANS indicates that in the absence of nucleotides (the state A·M) the arranged oscillators form an angle of about 42.5° with the thin filaments (Fig. 2a). In addition to the oriented fluorophore molecules, the muscle fiber also contains chaotically oriented dye molecules (see "Materials and Methods"). Since the chaotic orientation of fluorophores in a muscle fiber mainly results from oscillatory and rotary motions of myosin heads situated on a thin filament [29, 30], parameter *N* can be used as an index characterizing actin–myosin interaction [18, 28-31].

In the A·M state the relative amount of chaotically oriented oscillators *N* in the muscle fiber was about 30% ($N = 0.305 \pm 0.005$) (Fig. 2b). Correspondingly, the relative amount of arranged oscillators in the same fiber in the same structural state of actomyosin was about 70%. This suggests that in the A·M state the myosin heads possess high affinity to actin and are firmly bound with F-actin during formation of the so-called strong actin-myosin bond, which is essential for the generation of muscle force. The same conclusions on the actin–myosin interaction in the A·M state have been made earlier [18, 28, 29].

The Φ_E value significantly decreases in the presence of MgADP, and the axes of the probe emission dipoles turn to the axis of the muscle filament. The same motion

Table 1. Effect of 40-kDa actin-binding protein on the polarized fluorescence of myosin subfragment-1 (S1) labeled with 1,5-AEDANS in a muscle ghost fiber in absence and in presence of MgADP or MgATP

Nucleotides	S1	40 kDa	$P_{\parallel} \pm$ SEM	$P_{\perp} \pm$ SEM
–	+	–	0.410 ± 0.002	–0.022 ± 0.003
	+	+	0.366 ± 0.003	0.040 ± 0.002
MgADP	+	–	0.419 ± 0.004	–0.027 ± 0.003
	+	+	0.356 ± 0.004	0.077 ± 0.003
MgATP	+	–	0.322 ± 0.005	0.189 ± 0.006
	+	+	0.313 ± 0.005	0.266 ± 0.006

Notes: S1, myosin head subfragment-1; 40-kDa, actin-binding protein from the mussel; $P_{\parallel}$ and $P_{\perp}$, fluorescence polarization degrees with the fiber orientation parallel and perpendicular to the polarization plane of the exciting light, respectively. Six to ten ghost fibers were studied in each experiment.

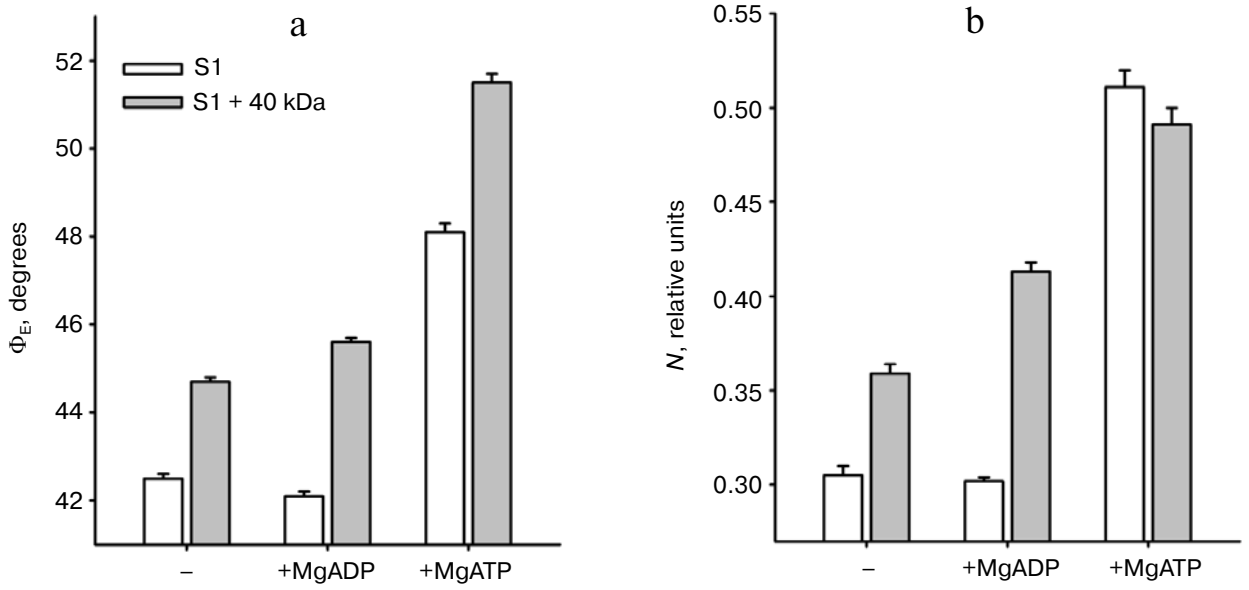

Fig. 2. Effect of MgADP and MgATP on the orientation Φ_E (a) and motility N (b) of myosin subfragment-1 labeled with 1,5-AEDANS (S1) in a muscle ghost fiber in the presence of 40-kDa actin-binding protein.

is evident from increase in fluorescence polarization degree when the fiber is oriented parallel to $P_\parallel$ (Table 1). It is known that addition of MgADP to thin filaments decorated with S1 [32] results in appearance of intermediate the A·M·ADP-like actomyosin state [33]. In this case, the relative amount of chaotically oriented oscillators N remains virtually unchanged in the presence of MgADP. Hence, myosin heads are firmly bound to F-actin to form the strong bond.

According to data of electron microscopy, myosin heads in the A·M state are at an angle of about 45° with thin filaments [34]. Most studies performed using fluorescence [35] and spin probes [32, 33] bound with Cys707 have shown slight, if any, change in slope of the myosin head caused by MgADP. This change likely reflects a "reverse motion" of the cross bridge [36]. Our experiments demonstrated a small, but significant, decrease of Φ_E in the presence of this nucleotide (Fig. 2a).

In the presence of MgATP the fluorescence polarization degree $P_\parallel$ decreases and $P_\perp$ increases when the fiber is parallel (Table 1). Φ_E increases from 42.1 to 48.1° (by 14%), suggesting that dipoles move from the muscle fibril axis. The relative amount of chaotically oriented oscillators N increases correspondingly from 0.302 to 0.511 (by 70%). The increase in N is indicative of considerable increase in amplitude of dipole oscillations of the probe, i.e. in mobility of myosin heads in thin filaments. Thus, a weak form of myosin head–actin bond appears in the presence of MgATP [29]. One can suppose that in the presence of MgATP different myosin states exist in combination, with predominance of A·M·ADP·Pi [37].

The 40-kDa protein conspicuously alters mobility and spatial position of the probe when modeling the strong and weak bonds between myosin subfragment-1 and actin (Fig. 2, a and b). In the absence of the nucleotides (A·M stage), the 40-kDa protein increased the fluorescent probe slope Φ_E from 42.5 to 44.7°. In this case the relative amount of chaotically oriented fluorophores N increases from 0.305 to 0.359 (by 18%), i.e. $P_\parallel$ decreases and $P_\perp$ increases (Table 1). Similar values of the polarization parameters were observed in the absence of this protein when modeling the weak actin–myosin interaction of the A·M·ADP·Pi stage (Table 1 and Fig. 2). Since the myosin head Cys707, a fluorescent probe target, is localized in the catalytic domain at the end of a short α-helix connecting the N-terminal subdomain with the "converter", a subdomain directly adjacent to the catalytic domain and undergoing the most prominent changes in orientation in the ATPase cycle [38], our data may indicate that the 40-kDa protein from the mussel dislodges the myosin head from the actin site responsible for the actin–myosin strong bond formation, thus weakening the actin–myosin interaction. This interpretation of the data is not the only one possible. Since the 40-kDa protein binds to actin [16], the changes in the state of actin–myosin interaction might result from actin conformation changes induced by binding with the 40-kDa protein. Further studies on the effect of this protein on the actin structural state in the ATP hydrolysis cycle will allow a more unequivocal conclusion.

Similar inhibitory effect of the 40-kDa protein on the actomyosin strong bond formation was observed in the presence of MgADP. Figure 2a shows that the Φ_E value in the presence of MgADP, which decreased in the absence of the 40-kDa protein from the mussel, increased from 42.1 to 45.6° in its presence. This is indicative of

Table 2. Effects of myosin subfragment-1 (S1) and MgADP and MgATP nucleotides on polarized fluorescence of 40-kDa actin-binding protein labeled with acrylodan

Nucleotides	S1	40 kDa*	$P_{\parallel} \pm$ SEM	$P_{\perp} \pm$ SEM
–	–	+	0.336 ± 0.003	0.353 ± 0.002
–	+	+	0.328 ± 0.003	0.384 ± 0.003
ADP	+	+	0.331 ± 0.002	0.386 ± 0.002
ATP	+	+	0.330 ± 0.003	0.376 ± 0.002

Notes: S1, myosin head subfragment-1; 40 kDa*, 40-kDa actin-binding protein labeled with acrylodan; $P_{\parallel}$ and $P_{\perp}$, fluorescence polarization degrees at fiber orientation parallel and perpendicular to the polarization plane of the exciting light, respectively. Eight to ten ghost fibers were studied in each experiment.

rotation of emission dipole axes away from the longitudinal axis of the muscle fiber, which is characteristic of the actin–myosin weak bond formation [29, 39]. The latter is also evident from changes of $P_{\parallel}$, $P_{\perp}$, and *N*. While $P_{\parallel}$ decreases and $P_{\perp}$ increases (Table 1), the relative amount of chaotically oriented fluorophore molecules *N* increases from 0.302 to 0.413 (by 37%). Hence, the 40-kDa protein inhibits formation of strong bonds between S1 and actin, favoring the weak bond formation. Interestingly, the same suggestion was made earlier from data on inhibition of actomyosin ATPase by calponin [5].

In the presence of MgATP, addition of the 40-kDa protein results in increase in Φ_E from 48.1 to 51.5° (Fig. 2a), decrease in $P_{\parallel}$, and increase in $P_{\perp}$ (Table 1). The relative amount of chaotically oriented fluorophore molecules *N* in these experiments was about 50%, which corresponds to the weak actomyosin bond, i.e. the state A·M·ADP·Pi. Comparison of these polarization characteristics with those obtained in experiments in the absence of the 40-kDa protein suggests that this protein has a little effect on formation of the weak bond between S1 and actin (Table 1). Our data also conform with early studies of molecular mechanisms of regulation of actomyosin interaction with vertebrate smooth muscle calponin [5].

In the present work we also studied motility and position of the 40-kDa protein on thin filaments of muscle ghost fiber by modeling different stages of the ATP hydrolysis cycle. The 40-kDa protein from the mussel modified with acrylodan was used in the experiments (see "Materials and Methods").

Decoration of thin filaments of a muscle ghost fiber with the 40-kDa protein modified with acrylodan results in appearance of polarized fluorescence of the ghost fiber (Fig. 1 and Table 2). Since the polarization degree $P_{\perp}$ (0.353 ± 0.002) was higher than $P_{\parallel}$ (0.336 ± 0.003), we suppose that either the fluorophore emission dipole axes are predominantly oriented perpendicular to the fiber axis, while the 40-kDa protein is parallel to the muscle fiber, or the dipoles are parallel to the fiber axis, while the 40-kDa protein is attached predominantly perpendicular to the thin filaments. Since the 40-kDa protein is a calponin-like protein [16], and the molecular masses of calponin (33 kDa) and 40-kDa protein are rather similar, and since calponins and calponin-like proteins share high homology [15, 40, 41], we suppose that linear sizes of these two proteins are also very similar. Since the calponin molecule is an elongate ellipsoid of revolution, 16.2 nm in length and 2.63 nm in diameter [42], such sizes most likely exclude a simultaneous contact of the calponin molecule with more than three adjacent actin monomers [43]. In solution, the stoichiometry of calponin–actin complexes is either 1 : 2 or 1 : 1 depending on ionic strength, with the ratio 1 : 1 at ionic strength less than 110 mM. Given the calponin size and low ionic strength of the experimental conditions, perpendicular orientation of the actin-bound calponin towards the thin filament [28] seems to be preferable, and we can conclude that the 40-kDa protein – under similar experimental conditions – binds to the thin filament in like manner.

One can see in Fig. 3a that in the absence of S1 the angle Φ_E is 52.9°, and the relative amount of chaotically oriented fluorophores *N* reaches 63%. Decoration of thin filaments with subfragment-1 increases the value of *N* (Fig. 3b) and increases the angle of emission dipole orientation from 52.9 to 55.5°(by 5%; Fig. 3a). This can be considered as an effect of S1 on the spatial position of the 40-kDa protein on the thin filaments of the muscle fiber. Since the fluorescent probe is firmly attached to the protein, we suppose that in absence of nucleotides S1 causes dislodgment (or rotation) of this protein towards the thin filament periphery. In this case, as evident from the increase in *N*, affinity of the 40-kDa protein to actin seems to decrease slightly.

Changes of actomyosin complex conformation during the ATPase cycle modeled with MgADP and MgATP influence the acrylodan polarization parameters $P_{\parallel}$, $P_{\perp}$, Φ_E, and *N*. MgADP increased Φ_E to 55.7° and MgATP decreased it to 54.7° (Fig. 3a). Interestingly, in the presence of MgATP Φ_E tends to the value characteristic of

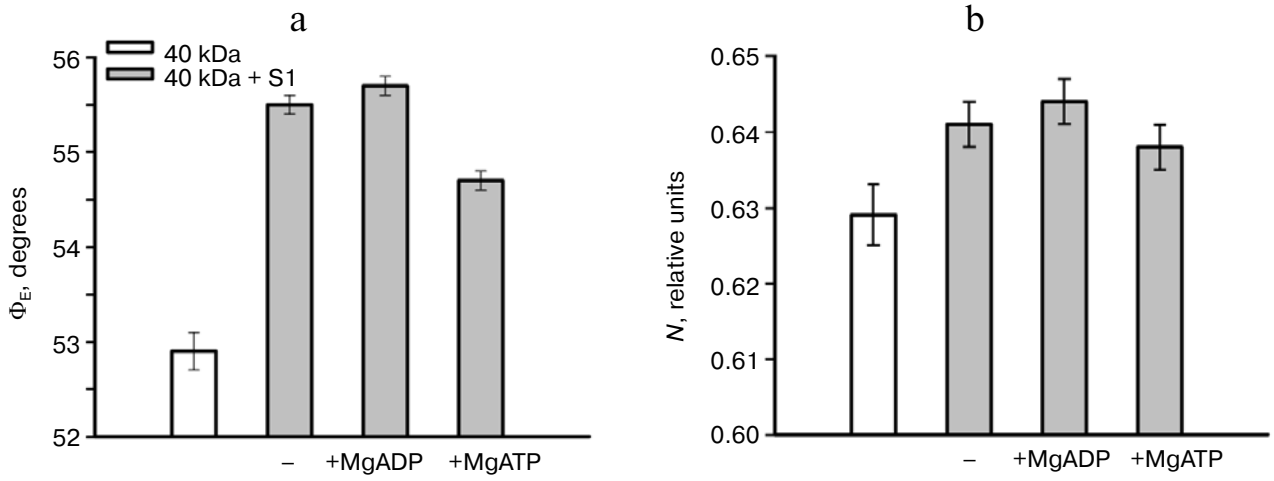

Fig. 3. Effect of different actomyosin complex states modeled with MgADP and MgATP on the orientation Φ_E (a) and motility (b) of the actin-binding protein labeled with acrylodan (40-kDa) in muscle ghost fibers.

thin filaments lacking subfragment-1. This may suggest that the weak form of actin–myosin interaction allows a partial restoration of the position of the 40-kDa protein on actin by dislodgment or rotation towards the thin filament axis.

Several mechanisms that might underlie the inhibitory effect of calponin on the MgATPase activity have been considered in the literature, including: calponin phosphorylation and dephosphorylation [4]; calponin binding with either Ca^{2+}-calmodulin [8] or caltropin [44].

Szymanski et al. [45] have shown that the binding of one molecule of recombinant chicken gizzard α-calponin (RαCaP) to every three to four actin monomers is sufficient for maximal inhibition of actin-activated rabbit skeletal muscle subfragment-1 ATPase. At this RαCaP/actin ratio, RαCaP does not interfere with S1 binding to F-actin. At higher concentrations RαCaP displaces S1 from F-actin, and a 1 : 1 RαCaP–actin monomer complex is formed. These data suggest competition between myosin and calponin for common binding sites on the actin monomer, which meets our supposition made above. Since calponin binds to the actin Glu334 residue [46], which is localized in the site responsible for the strong binding with myosin head [47], we suppose that the binding of calponin to this site is one of the causes of inhibition of ATPase by this protein. It is worth noting that the studied 40-kDa protein not only inhibits MgATPase like calponin does, but it also does it in a similar way, via inhibition of strong bond formation, as reported earlier for smooth muscle calponin [5]. Earlier, we found that mussel tropomyosin has no effect on polarization parameters in experiments with the 40-kDa protein (data not shown), which also makes the latter kin to calponin. It is known that tropomyosin does not interfere with calponin binding to actin [48], its inhibitory effect on MgATPase [49], and motion of actin filaments in artificial motility systems [9, 50]. Thus, the data presented suggest that the 40-kDa protein from the mussel can alter the character of actin–myosin interaction in the ATP hydrolysis cycle by influencing affinity of myosin to actin and changing its own position on thin filaments. This protein probably competes with S1 for site(s) of strong binding of myosin head on actin, thus inhibiting the actin–myosin strong bond formation that is essential for force generation. The 40-kDa actin-binding protein apparently has many of the properties characteristic of smooth muscle calponin.

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