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Effect of Nucleotides on the Orientation and Mobility of Myosin Subfragment-1 in Ghost Muscle Fiber

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Abstract—Using polarization fluorimetry, the orientation and mobility of 1,5-IAEDANS specifically bound to Cys707 of myosin subfragment-1 (S1) were studied in ghost muscle tropomyosin-containing fibers in the absence and in the presence of MgADP, MgAMP-PNP, MgATPγS, or MgATP. Modeling of various intermediate states was accompanied by discrete changes in actomyosin orientation and mobility of fluorescent dye dipoles. This suggests multistep changes in the structural state of the myosin head during the ATPase cycle. Maximal differences in the probe orientation by 4° and its mobility by 30% were found between actomyosin states in the presence of MgADP and MgATP. It is suggested that interaction of S1 with F-actin induces nucleotide-dependent rotation of the whole motor domain of the myosin head or only the dye-binding site and also change in the head mobility.

Key words: muscle contraction, ATP analogs, intermediate states of actomyosin, conformational changes of myosin subfragment-1, fluorescence polarization

It is well recognized that ATP hydrolysis by myosin is the energy source for muscle contraction. During the ATPase cycle the head of myosin molecule known as myosin subfragment-1 (S1) undergoes several conformational changes detected in kinetic studies [1-4]. Structural states of myosin can be attributed to two groups typical for weak and strong binding forms with actin [5-7]. The group of weak actin-binding (actin-detached) states includes actomyosin·ATP (A·M*·ATP state) and actomyosin·ADP·P_i (A·M**·ADP·P_i), whereas the group of strong-binding states includes actomyosin without nucleotide (A·M state) and in the presence of MgADP (A·M^·ADP) [8], where A is actin, and M, M*, M**, and M^ represent intermediate conformational states of myosin.

It is generally accepted that the development of tension generated in the process of muscle fiber contraction occurs during transition of a weak binding state of the cross-bridges to their strong binding state (see for review [9]). Earlier it was suggested that binding of myosin head to actin filaments is accompanied by its rotation during the ATP hydrolysis cycle, and this provides the relative sliding of thin and thick filaments [10]. However, electron microscopy and X-ray diffraction studies demonstrated that in the weak binding state the cross-bridges are more or less disordered, whereas in the strong binding state they are well ordered [11, 12]. Studies of three-dimensional structure of S1 [13] indicate that orientation changes involve large alterations in the regulatory domain of the myosin head. The authors suggest that during force generation the position of motor (catalytic) domain of the myosin head remains unchanged, and large orientation changes involve the whole regulatory domain of the myosin head ("lever arm"). These orientation changes occur due to significant conformational changes in the motor domain of the myosin head [13, 14]. This is now the most popular hypothesis; it is supported by the results of several experimental studies employing various spectral methods [9]. However, data obtained by these methods may also be alternatively interpreted as rotation of the myosin head around actin filament [15, 16].

It was earlier shown that polarization fluorimetry is a highly sensitive method applicable for studies of conformational changes of myosin during muscle contraction [15-22]. Using this method, we have investigated changes

Abbreviations: A·M) actomyosin; DTT) dithiothreitol; 1,5-IAEDANS) N-iodoacetyl-N′-(5-sulfo-1-naphthyl)ethylene diamine; S1) myosin subfragment-1; AMP-PNP) 5′-adenylylimidodiphosphate; ATPγS) adenosine 5′-O-(3-thiotriphosphate).

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occurring in the structural state of the myosin head during modeling of various intermediate states of actomyosin (A·M, A·M^·ADP, A·M′·ADP, A·M*·ATP, and A·M**·ADP·P_i). These states of the ATP hydrolysis cycle were modeled by decorating S1 thin filaments with MgADP, MgAMP-PNP, MgATPγS, or MgATP or without nucleotide. The Cys707 residue of S1 was modified by the fluorescent probe 1,5-IAEDANS. Interaction of S1 with F-actin caused nucleotide-dependent structural changes in the myosin head; this apparently involves rotation of the motor domain or dye binding site and changes in myosin head mobility.

MATERIALS AND METHODS

Myosin was isolated from rabbit skeletal muscles [23]. S1 was obtained by treatment of myosin with α-chymotrypsin at 25°C for 10 min [24]. The Cys707 residue of myosin subfragment-1 was modified with 1,5-IAEDANS as described earlier [18]. The modification degree calculated using molar absorbance coefficient of 6100 $M^{-1}\cdot cm^{-1}$ at 336 nm [25] was 0.9-0.95 (n = 5). Tropomyosin was obtained as described earlier [26].

Glycerinated fibers were obtained from rabbit back muscles by the method of Szent-Gyorgyi [27]. Thick filament proteins, troponin, and tropomyosin were removed by incubating a single glycerinated muscle fiber in solution containing 0.8 M KCl, 1 mM $MgCl_2$, 10 mM ATP, 67 mM phosphate buffer, pH 7.0, for 1.5 h. Such fibers (so-called ghost fibers) contain more than 80% actin [28]. Ghost fibers were reconstituted with S1 and tropomyosin by their incubation with the ghost fiber in solution containing 10 mM KCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT), 10 mM Tris-HCl buffer, pH 6.8, and 1-2.5 mg/ml protein [29]. Unbound protein was removed by the same solution but without protein. Protein composition of muscle fiber and S1/actin molar ratio were determined by SDS-PAGE by the method of Laemmli [30] followed by subsequent gel densitometry (UltroScan XL, Pharmacia LKB, Sweden).

Polarized fluorescence of 1,5-IAEDANS–actin emission was recorded at 480-550 nm after excitation at 365 ± 5 nm. Measurements were carried out in solution containing 10 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 6.7 mM phosphate buffer, pH 7.0, in the absence of nucleotides and in the presence of 2.5 mM ADP, 15 mM AMP-PNP, 25 mM ATPγS, or 5 mM ATP. In the absence of the nucleotides and in the presence of ADP, AMP-PNP, ATPγS, and ATP the molar ratio S1/actin was 1 : 5 (±2), 1 : 5 (±2), 1 : 8 (±2), 1 : 12 (±2), and 1 : 14 (±2), respectively. Four intensities of polarized fluorescence of muscle fiber were registered by fiber orientation parallel ($_{\parallel}I_{\parallel}$, $_{\parallel}I_{\perp}$) and perpendicular ($_{\perp}I_{\perp}$, $_{\perp}I_{\parallel}$) to the polarization plane of the exciting light. Ratio of polarized fluorescence was defined as:

$$P_{\parallel} = (_{\parallel}I_{\parallel} - _{\parallel}I_{\perp})/(_{\parallel}I_{\parallel} + _{\parallel}I_{\perp}),\ P_{\perp} = (_{\perp}I_{\perp} - _{\perp}I_{\parallel})/(_{\perp}I_{\perp} + _{\perp}I_{\parallel}).$$

The experimental data were analyzed using the model-dependent method [31, 32] and assuming that in the muscle fiber there is a fraction of helically located fluorophores (characterized by orientation angles of oscillator absorbance and emission of Φ_A and Φ_E, respectively), and also a fraction of disordered fluorophores (N). Since in all experiments changes in Φ_A correspond to changes in Φ_E, the formers are not shown. The statistical significance of changes was evaluated by Student's criterion.

RESULTS AND DISCUSSION

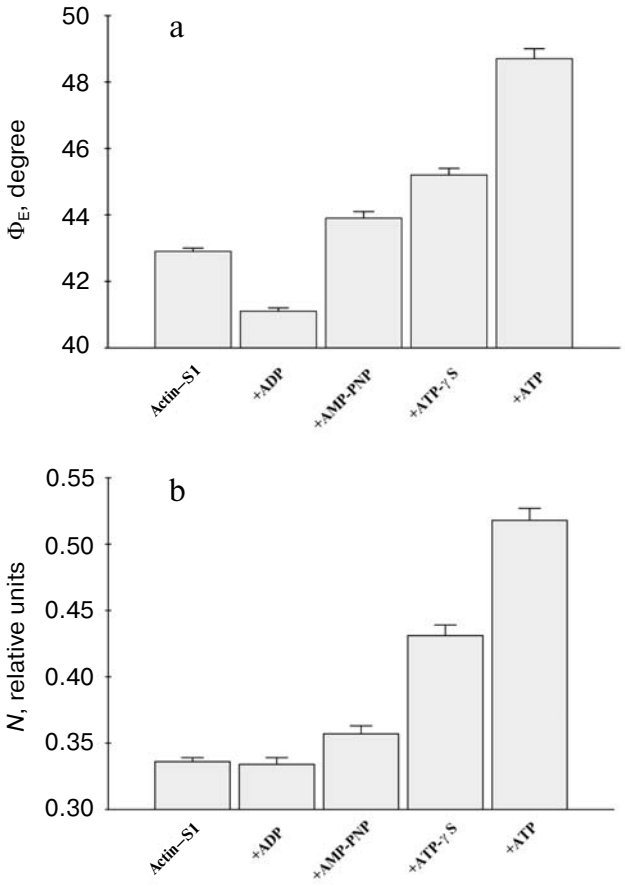
Earlier it was shown that polarized fluorescence from 1,5-IAEDANS specifically bound to Cys707 of the motor domain of myosin head molecule could be successfully used for structural analysis of the myosin head during muscle contraction [15-22].

Decoration of ghost fiber thin filaments with myosin subfragment-1 modified with 1,5-IAEDANS caused appearance of fiber polarized fluorescence. In accordance with previously published data [21], the polarized fluorescence degree was high during parallel ($P_{\parallel}$) orientation of the fiber to the light polarization plane ($P_{\parallel}$ = 0.412 ± 0.001) and low during perpendicular ($P_{\perp}$) fiber orientation to the light polarization plane ($P_{\perp}$ = 0.025 ± 0.002). This suggests that dye absorption and emission dipoles are preferentially oriented parallel to the fiber axis [17, 18, 21].

In the absence of nucleotides (A·M state) heads of myosin molecules are well oriented; they form a sharp angle of ~45° with thin filament [33]. Analysis of fluorescence of S1 labeled with 1,5-IAEDANS indicates that in the absence of nucleotide in the muscle fiber there are two fluorophore populations: a fraction of randomly positioned oscillators and a fraction of oscillators oriented at the angle of 43° (figure). It is possible that the ordered dye molecules are positioned parallel to the motor domain of the myosin head molecule.

Since the fraction of randomly oriented oscillators appears mainly due to oscillation and rotation movements of the myosin molecule head [19, 21], the N parameter can be used for characterization of actin–myosin interaction [17-21]. Under our experimental conditions, in the absence of nucleotide relative number of randomly located 1,5-IAEDANS oscillators was less than 40% (N = 0.336 ± 0.003, figure). This suggests that myosin heads are strongly bound to actin [17, 18, 21].

The figure shows that MgADP, MgAMP-PNP, MgATPγS, and MgATP had significant influence on Φ_E and N values. Changes in the polarization parameters were statistically significant ($p < 0.05$) and reversible. Replacement of solution containing MgATP for a



Effects of MgADP, MgAMP-PNP, MgATPγS, and MgATP on orientation (a) and mobility (b) of myosin heads in ghost muscle fiber; Φ_E is the mean of an angle between the fiber axis and emission oscillator of 1,5-IAEDANS; *N* is the relative number of disordered fluorophores. Data represent mean of 80 determinations obtained using 16 fibers. Standard errors for Φ_E and *N* did not exceed 0.3° and 0.01, respectively. There were statistically significant changes in Φ_E and *N* values ($p < 0.05$) between actin–S1, actin–S1–ADP, actin–S1–AMP-PNP, actin–S1–ATPγS, and actin–S1–ATP complexes

nucleotide-free solution almost completely restored the initial values of the polarization parameters. This suggests that changes in polarized fluorescence of the ghost fiber were mainly determined by the nucleotide effect on structural state of myosin in the S1–actin complex.

At low nucleotide concentrations the structural state of the S1 population could be heterogeneous. To avoid experimental errors originating from such heterogeneity, we used the optimal concentration of each ATP analog. This approach revealed that the increase in nucleotide concentrations was accompanied by an increase in nucleotide effect. Maximal changes of polarization parameters were observed at 1.5, 10, 18, and 2.5 mM of ADP, AMP-PNP, ATPγS, and ATP, respectively. Further increase in concentrations of these nucleotides up to 3, 15, 25, and 7 mM, respectively, did not cause further changes in the polarization parameters. This suggests structural state homogeneity of S1. The figure shows results of experiments in the presence of 2.5 mM ADP, 15 mM AMP-PNP, 25 mM ATPγS, or 5 mM ATP (i.e., under conditions of homogenous structural state of the S1–1,5-IAEDANS–nucleotide population). Similar concentrations of ATP analogs were used earlier [34, 35].

The polarized fluorescence of 1,5-IAEDANS is determined by the structural state of myosin head [15-21]. Over a wide range of molar ratios of myosin/actin it did not depend on S1 concentration in this fiber [19, 21]. In our experiments increase in the molar ratio S1/actin from 1 : 20 to 1 : 3 did not influence the polarized fluorescence parameters in the absence and in the presence of ATP analogs. The results shown in the figure were obtained in the range of S1/actin molar ratios from 1 : 14 to 1 : 5, i.e., under conditions when the polarization parameters did not depend on concentration of myosin heads. Consequently, differences in orientation or mobility of the dye under modeling of various intermediate states of myosin ATPase reactions reflect different structural states of myosin heads in the muscle fiber.

Addition of MgADP to S1-decorated thin filaments or to rigor muscle fibers [36, 37] resulted in appearance of an intermediate state of actomyosin close to the state A·M^·ADP [35]. According to electron microscopy data in this state the molecules of myosin heads form an angle of ~45° with the thin filaments [33]. Most studies employing fluorescent [9] and spin [35, 37] labels bound to Cys707 demonstrated weak (if any) effect of MgADP on the tilt of the myosin head. It is suggested that this change reflects "reversed movement" of the cross-bridge [38]. Under our experimental conditions we also found small (but significant) reduction in Φ_E by $1.8 \pm 0.1°$ in the presence of this nucleotide (figure).

The fluorescent probe, 1,5-IAEDANS, is known to be covalently bound to the head of the myosin molecule [25]; consequently, changes in dye orientation can be considered as changes in orientation of the myosin cross-bridge [17, 18].

However, this is not the only possible interpretation. According to modern notions, modeling of various stages of the ATPase cycle is accompanied by significant conformational changes in the motor domain of the myosin head involving movements of some subdomains; this results in "lever arm" (regulatory domain) rotation and force generation [39]. Amino acid residue Cys707 involved in binding of the fluorescent probe is located in the motor domain, in the end of a short α-helix linking the N-terminal subdomain with the "converter" subdomain flanking the regulatory domain and undergoing the most pronounced orientation changes during the ATPase cycle. So, it is possible that orientation and mobility of the Cys707 residue (and the fluorescent probe attached to this residue) may be significantly changed in various intermediate states of the ATPase cycle. For example,

MgADP might cause rotation of Cys707 together with the "converter" (change in orientation in the motor domain of the myosin head) without changes in the position of the motor domain relative to actin.

Since there are no data on rotation of the whole myosin molecule but there are data on subdomain movement in the motor domain [39], the results shown in the figure may be more easily interpreted as changes in azimuthal orientation of the motor domain [16] or part(s) of this domain containing the fluorescent dye.

According to this interpretation changes in 1,5-IAEDANS orientation found in the presence of MgADP could be explained by deviation of the motor domain (or its dye containing part) from the position typical for rigor to long axis of the muscle fiber. The relative number of disordered 1,5-IAEDANS oscillators was less than 40% ($N = 0.334 \pm 0.005$, see the figure). This suggests strong binding of the myosin head to actin in the actin–S1–ADP complex.

MgAMP-PNP, MgATPγS, and MgATP also cause changes in Φ_E and N (figure). However, under these conditions these parameters increased.

It is known that MgAMP-PNP attenuates myosin interaction with actin. In the presence of this ATP analog, myosin heads form with actin strong binding [40]. The actomyosin–AMP-PNP complex has mechanical [41], structural [42, 43], and orientation [44] properties that are intermediate between rigor and relaxation. Evidence exists that in the presence of this ATP analog there are two populations of myosin heads in the muscle fiber: fractions of actin-bound and actin-unbound myosin heads. The orientation of the fraction of actin-unbound myosin heads is totally disordered, whereas organization of the fraction of actin-bound myosin heads is strictly ordered [35].

In this study experiments were carried out at low ionic strength. Under these conditions equilibrium between these two fractions shifts towards the fraction of actin-bound myosin heads [2]. So we suggest that in the presence of MgAMP-PNP polarized fluorescence of the muscle fiber can be attributed to the actin-bound myosin heads (1,5-IAEDANS–S1). The figure shows that MgAMP-PNP caused statistically significant increase in Φ_E ($p < 0.05$). Consequently, in the actin–S1–AMP-PNP complex the tilt angle of the motor domain (or a dye-containing fragment) increased, thus indicating rotation of the fluorescent probe by $1.0 \pm 0.1°$ towards the muscle fiber axis (i.e., in the direction of such label orientation which is typical for weak myosin binding with actin) [21]. In the presence of MgAMP-PNP the N value was $6.0 \pm 1.7\%$ higher than in the presence of MgADP (figure). This suggests formation of weak binding between myosin heads and actin [17, 18]. Apparently, the actin–S1–AMP-PNP complex exhibits properties typical for weak binding of myosin with actin. This is consistent with conclusions based on results of studies using a spin label attached to Cys707 [35]. The authors concluded that in the actomyosin stated defined as A·M′·ADP, MgAMP-PNP mimics weaker binding of myosin heads than MgADP does.

Marked increases in Φ_E and N parameters were also found in the presence of MgATPγS or MgATP. Evidence exists that in the presence of MgATP there are several various myosin states, where M**·ADP-P_i predominates [45]. Results of mechanistic and spectroscopic studies suggest that the state of myosin head in the presence of MgATPγS resembles an intermediate state of the ATPase state close to M*·ATP [45-48]. In our experiments, MgATPγS and MgATP caused significant increase in Φ_E and N values. This suggests weak binding of myosin heads to actin in the presence of these nucleotides. However, in the presence of MgATPγS, Φ_E and N were lower than in the presence of MgATP. For example, in the presence of MgATPγS, the Φ_E and N values were higher by $2.3 \pm 0.2°$ and $28.2 \pm 2.3\%$, respectively, than in the absence of nucleotide, whereas in the presence of MgATP these parameters were higher by $4.9 \pm 0.3°$ and $54.2 \pm 2.6\%$, respectively (than in the absence of nucleotide). These results suggest similar structural states of myosin heads in the actin–S1–ATPγS and actin–S1–ATP complexes. In the presence of ATPγS the structural state of actomyosin is between A·M′·ADP and A·M**·ADP·P_i, whereas in the presence of MgATP actomyosin is preferentially in the state A·M**·ADP-P_i.

Thus, the data obtained indicate that in the actin–S1–nucleotide complex the structural states of myosin heads significantly differ in the weak and strong binding states. Each of these groups of binding is characterized by intermediate states of actomyosin differing in mobility of the myosin head and orientation of the motor domain or the dye-containing fragment of this domain. In these actomyosin intermediate states reduction of tilt angle of dye oscillators and mobility of the myosin heads decreased in the following order: A·M**·ADP·P_i (in the presence of MgATP) > A·M*·ATP (in the presence of MgATPγS) > A·M′·ADP (in the presence of MgAMP-PNP) > A·M (in the absence of nucleotide) > A·M^·ADP (in the presence of MgADP).

Analysis of three-dimensional structure of S1 suggests that maximal changes in orientation of myosin head regulatory domain (the "lever arm") occur during myosin head transition from the structural state M**·ADP·P_i to M^·ADP [9]. Results of the present study suggest that during the transition from the weak actomyosin binding state to the strong binding state the motor domain of the myosin head or its part (the fragment of α-helix linking N-terminal subdomain with the "converter") undergoes rotation by 4° (figure). Similar changes have recently been found in the whole regulatory domain [20]. Some evidence exists that during muscle contraction these regions of the myosin head rotate as a "rigid body" [49]. It is possible that during the ATPase cycle the region con-

taining Cys707 is also rotated in the motor domain of the myosin head together with "converter" and the regulatory domain.

We have recently demonstrated that each intermediate structural state of the myosin head corresponds to certain orientation and mobility of monomer actin in thin filaments [50]. It is possible that structural rearrangements of myosin reach actin and cause changes in monomer actin in thin filaments.

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